Relationship between Activity, D1 Loss, and Mn Binding in Photoinhibition of Photosystem II†

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ABSTRACT: Photoinhibition of photosystem II (PSII) activity and loss of the D1 reaction center protein were studied in PSII-enriched membrane fragments in which the water-splitting complex was inhibited by depletion of either calcium or chloride or by removing manganese. The Ca^{2+}-depleted PSII was found to be the least susceptible to inhibition by light as reported previously (Krieger, A., and Rutherford, A. W. (1997) *Biochim. Biophys. Acta 1319*, 91−98). This different susceptibility to light was not reflected in the extent of D1 protein loss. In Mn-depleted PSII the loss of activity and the loss of the D1 protein were correlated, while in Cl^-- and Ca^{2+}-depleted PSII, there was very little loss of the D1 protein. The production of free radicals and singlet oxygen was measured by EPR spin-trapping techniques in the different samples. $^1O_2$ and carbon-centered radicals could be detected after photoinhibition of active PSII, while hydroxyl radical formation dominated in all of the other samples. In addition, photoinhibition of PSII was investigated in which the functional Mn cluster was reconstituted (i.e., photoactivated). As expected this led to a protection against photoinhibition. When the photoactivation procedure was done in the absence of Ca^{2+} no activity was obtained although a nonfunctional Mn cluster was formed. Despite the lack of activity the binding of Mn partially protected against the loss of D1. These data demonstrate that, during photoinhibition, the extent of D1 loss is neither affected by the water-splitting activity of the sample nor correlated to the kinetics of PSII activity loss. D1 loss seems to be independent of the chemical nature of the reactive oxygen species formed during photoinhibition and seems to occur only in the absence of Mn. It is proposed that Mn binding protects against D1 loss by maintaining a protein structure which is not accessible to cleavage.

Photoinactivation of photosystem II (PSII)[1] occurs when PSII is exposed to more light than is required for saturating photosynthetic electron flow (*1*−*4*). Different processes are associated with photoinhibition: the activity of PSII is inhibited and the D1 reaction center protein is degraded. These two processes occur with different kinetics (e.g., refs *5* and *6*). The D1 loss is temperature-dependent (*7*) and slower than the inhibition of PSII activity.

Depending on the experimental conditions, different mechanisms of photoinactivation seem to exist and can be investigated separately in vitro. The mechanism of photodamage depends on the site within PSII where electron transport becomes limited under excess light. One route of photoinhibition discussed in the literature is the so-called donor-side-induced photoinhibition. According to this model, impairment of the water-splitting complex leads to the light-induced accumulation of highly oxidizing species such as $P680^+$ and/or $TyrZ^+$ which promote D1 degradation (e.g., refs *6* and *8*−*11*). The other route is the so-called acceptor-side-induced photoinhibition and occurs in PSII with a functional donor side mostly under strong reducing conditions (e.g., refs *12* and *13*). In this process, excess light leads to a complete reduction of the plastoquinone pool. Q_A seems to become double reduced, and a long-lived chlorophyll triplet state becomes detectable (*13*, *14*). The long-lived chlorophyll triplet can react with $^3O_2$ to form $^1O_2$ which is thought to be the species responsible for the photodamage (*13*, *15*).

Toxic oxygen species were proposed to be involved in triggering the degradation of the D1 protein (*12*, *16*−*19*). During photoinhibitory treatment the production of $^1O_2$ was measured by phosphorescence (*20*) and chemical trapping (*21*). In isolated thylakoid membranes, the formation of $^1O_2$

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[1] Abbreviations: Chl, chlorophyll; D1 protein, reaction centre-binding protein; DCPIP, 2,6-dichlorophenol-indophenol; DMPO, 5,5-dimethyl-1-pyrrolin-*N*-oxide; DMPO−OH, hydroxyl radical adduct of DMPO; DMPO−R, Carbon centred radical adduct of DMPO; DPC, 1,5-diphenylcarbazide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PSII, photosystem II; Q_A, primary quinone acceptor in PSII; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TyrZ, redox active tyrosine in PSII.

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and other free radicals has also been monitored with spin-trapping EPR spectroscopy (*22*, *23*). $^1O_2$ was shown to be involved in acceptor-side-induced photoinhibition using this technique. In contrast, there was no detectable singlet oxygen production during donor-side-induced photoinhibition (*23*). In this way, the observation of $^1O_2$ production can be regarded as a characteristic of acceptor-side-induced photoinhibition. Similarly, the two pathways of photoinhibition are distinct in the chemical nature of the dominant free radical observed: oxygen-centered, mainly hydroxyl radicals accompany donor-side-induced photoinhibition, while (in addition to $^1O_2$) carbon-centered radicals dominate during acceptor-side-induced photoinhibition (*23*).

The relevance of the above in vitro studies to the mechanism of photoinhibition in vivo has not been fully investigated, although there are indications for the occurrence of both acceptor-side-induced and donor-side-induced pathways of damage in intact leaves (*24*−*26*).

PSII is synthesized without the Mn cluster, and subsequently, the assembly of the Mn cluster occurs in a light-dependent process (so-called photoactivation) (*27*−*31*). Prior to and during the photoactivation process, PSII is expected to be particularly vulnerable by light. However, the high midpoint potential of the Q_A/Q_A^- couple in PSII prior to photoactivation may decrease this susceptibility to photodamage (*32*). It has also been suggested that inactive PSII centers lacking the Mn cluster occur in vivo (*2*, *3*, *33*).

In a recent study, Krieger and Rutherford (*34*) compared the susceptibility of electron transfer to photoinhibition in PSII samples which were inhibited by Ca^{2+}, Cl^-, or Mn depletion. Significant differences in their susceptibility to light-induced inhibition were observed, with Ca^{2+}-depleted PSII being much less vulnerable than the other cases. Since the different types of inhibition of the water-splitting complex have different effects not only on the donor side but also on the acceptor side of PSII, several different explanations for the differential susceptibility could only be hypothesized. In the present work we have extended this study. We investigated the effects of photoinhibitory illumination on PSII activity, on the stability of the D1 protein, and on free radical formation in Ca^{2+}-, Cl^--, and Mn-depleted PSII-enriched membrane fragments. Additionally, we performed photoinhibition experiments on photoactivated samples and samples subjected to the same reactivation procedure but in the absence of Ca^{2+}.

MATERIALS AND METHODS

Preparation of PSII Samples. PSII-enriched membrane fragments from spinach were prepared essentially as described in ref *35* with modification as described in ref *36*. The activity of these samples was about 500 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}.

Ca^{2+} depletion was performed by incubation of PSII samples at room temperature for 5 min in room light (10−12 μmol of quanta m^{-2} s^{-1}) in a buffer containing 300 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, and 25 mM succinic acid (pH 4.5). Following this, the same medium was added but with 5 mM EGTA and 80 mM MES instead of succinic acid in order to readjust the pH to 6.5. The residual activity after this treatment was very low (20−50 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}). Re-addition of $CaCl_2$ restored the activity to 70%−80% of the control sample.

Cl^- depletion was performed by alkaline pH treatment in room light as described in ref *37*. In this treatment, sucrose "Superpure" (BDG Laboratory Supplies, England) was used to reduce the contamination from chloride. PSII samples were incubated for 30 s at pH 10, which resulted in a decrease of their activity to 50 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}, measured at pH 6.5. By re-addition of NaCl, 80% of the original activity was obtained.

Mn depletion was carried out by incubating PSII samples in a buffer containing 5 mM NH_2OH, 400 mM sucrose, 15 mM NaCl, and 50 mM Mes (pH 6.5) for 1 h in the dark on ice. This treatment was followed by two washes in the same buffer without NH_2OH. The residual activity after NH_2OH treatment was low (less than 5% of the activity prior to the treatment).

Photoactivation. Photoactivation was done according to ref *30* by incubating NH_2OH-treated PSII samples for 30 min at room temperature at 30 μmol of quanta m^{-2} s^{-1} white light in a buffer containing 400 mM sucrose, 15 mM NaCl, 50 mM Mes (pH 6.5), 6 μM DCPIP, and, depending on the actual experiment, 100 μM $MnCl_2$ and/or 50 mM $CaCl_2$. After photoactivation, samples were washed twice in the same buffer (without DCPIP, $MnCl_2$, $CaCl_2$) to remove unbound Mn.

Photoinhibitory Treatment. Photoinhibition was performed by illuminating the samples (150 μg Chl/mL) at room temperature with white light, intensity 1100 μmol of quanta m^{-2} s^{-1}. A water filter was used for heat protection, and the samples were stirred during the treatment. In the case of Cl^--depleted PSII, photoinhibition was performed in a NaCl-free buffer.

PSII Activity Measurements. Photosynthetic oxygen evolution was measured with a Clark-type oxygen electrode (Hansatech) at pH 6.5, using 1 mM ferricyanide and 0.5 mM *p*-phenylbenzoquinone as electron acceptors.

As a measurement of PSII activity, the electron transfer from water or DPC, as artificial electron, to DCPIP was measured at 620 nm with a Philips PU 8750 UV/VIS spectrophotometer equipped with side illumination using saturating light (6500 μmol of quanta m^{-2} s^{-1}). The assay medium consisted of 300 mM sucrose, 15 mM NaCl, 50 mM Mes (pH 6.5), 35 μM DCPIP, and, when used, 1 mM DPC. The chlorophyll concentration was 5 μg/mL for all samples.

Gel Electrophoresis and Immunoblotting. Degradation of the D1 protein was followed by immunoblotting as described in ref *55*. SDS−PAGE was performed on a 12%−17% linear acrylamide gradient gel containing 6 M urea. The resolved proteins were electroblotted and identified by using a polyclonal antibody raised against the D1 protein. Densitometric analysis of the immunodecorated blots was performed using the Stratagene Eagle Eye II video system.

EPR Measurements. Singlet oxygen was determined according to the method of Lion et al. (*38*, *39*) modified for measuring with thylakoid membranes (*22*), in samples containing 10 mM TEMP, by measuring the EPR absorption of the stable nitroxide radical (TEMPO) which is produced from the reaction between $^1O_2$ and TEMP. To avoid the production of *N*-hydroxylamine from the nitroxide, samples were extracted into ethyl acetate and oxygenated in the presence of a catalytic amount of PbO_2 before EPR spectroscopy, as described earlier (*22*). Spin-trapping EPR spectroscopy was performed in the presence of 67 mM

DMPO (5,5-dimethyl-1-pyrrolin-*N*-oxide) (for reviews see refs *40* and *41* and references therein). Neither TEMP nor DMPO affected the photosynthetic activity of thylakoids at the applied concentrations (data not shown). Illumination of the spin traps only (i.e., in the absence of thylakoids but under the same conditions as in the experiments with thylakoids) did not result in the formation of an EPR signal.

EPR spectra were measured with a Bruker ECS-106 spectrometer. X-band spectra were recorded at room temperature with 9.45 GHz microwave frequency, 16 mW microwave power, and 100 kHz modulation frequency, as described earlier (*42*). To ensure comparative EPR quantitation, all spectra of spin traps in thylakoid membranes were measured under identical experimental conditions regarding both the samples (volume, concentration, uniform sample holders, and the same central position in the cavity) and the EPR parameters (constant gain and modulation). Data collection and all calculations were performed using the spectrometer's ECS-106 data acquisition software.

Atomic Absorption Measurements. Mn determinations were made at 279.5 nm using an atomic absorption spectrometer (Perkin-Elmer 2280). Before this measurement, samples which contained 150 μg of Chl mL^{-1} were washed twice in order to remove loosely bound Mn, and the pellet was resuspended in the usual buffer and diluted first 1:1 in 0.4% HNO_3. The samples were then diluted by 1:20 in 0.4% HNO_3. Aliquots (10 μL) of this final solution were then pipetted into the graphite furnace. Calibration was done by using a standard Mn solution (Sigma).

RESULTS

Figure 1A shows the loss of PSII activity during a time course of photoinhibition with white light of an intensity of 1100 μmol of quanta m^{-2} s^{-1} in control, Mn-, Ca^{2+}-, and Cl^--depleted PSII-enriched membrane fragments. PSII activity was measured as the reduction of DCPIP. In the case of Ca^{2+}-depleted PSII, 50 mM $CaCl_2$ and, in the case of Cl^--depleted PSII, 15 mM NaCl were added to the medium to restore water-splitting activity of the sample after the photoinhibitory treatment. The PSII activity of Mn-depleted PSII was measured in the presence of the artificial electron donor DPC. Photoinhibition of samples inactive in water splitting results in a faster loss of activity compared to controls. The extent of photodamage was higher in Cl^--depleted than in Ca^{2+}-depleted PSII, in accordance with an earlier report (*34*). Figure 1A shows that Mn- and Cl^--depleted PSII were susceptible to a similar extent to light. It was previously observed that Cl^--depleted PSII was more susceptible to inhibition than Mn-depleted PSII, but in the present study we systematically found no difference between these two types of material.

Figure 1B shows the amount of D1 protein during photoinhibition in control, and Mn-, Ca^{2+}-, and Cl^--depleted PSII-enriched membrane fragments. We focused on the determination of total D1 loss, because we were not able to detect specific fragments of the degraded D1 protein. The detection of specific degradation fragments is easier in more purified PSII preparations, for example, core particles (*3*). As an example, an immunoblot is shown in Figure 1C; the samples were subjected for 10 and 25 min to the photoinhibitory illumination. In our photoinhibition experiments,

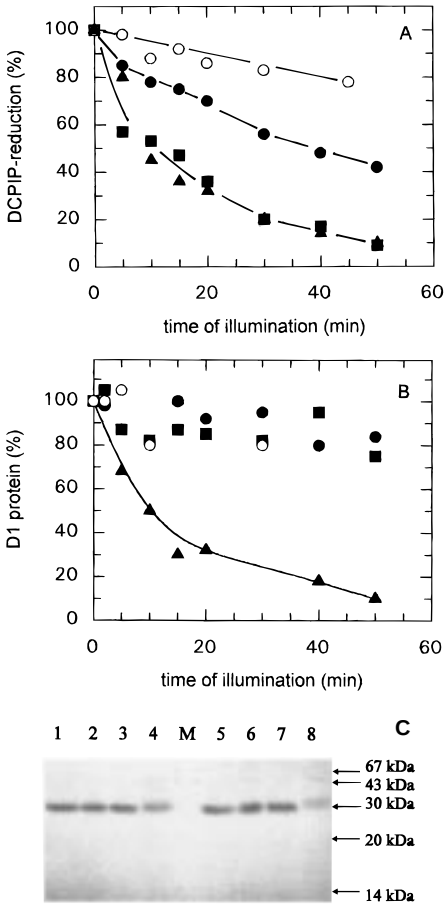

FIGURE 1: Photoinhibition of Cl^-- (closed squares), Ca^{2+}-depleted (closed circles), and Mn-depleted (closed triangles) and active (open circles) PSII-enriched membrane fragments. The samples were incubated in white light (1100 μmol of quanta m^{-2} s^{-1}) at room temperature. (A) Photoinhibition of PSII activity. The rate of DCPIP reduction was measured using either water (active or Ca^{2+}- or Cl^--depleted PSII) or 1 mM DPC (Mn-depleted PSII) as the electron donor. In the case of Ca^{2+}- or Cl^--depleted PSII, either 50 mM $CaCl_2$ or 20 mM NaCl was added prior to the activity measurement to restore water-splitting activity. The activities prior to photoinhibition were the following: control (active) 934 μmol of $DCPIPH_2$, Cl^--depleted 700 μmol, Ca^{2+}-depleted 654 μmol, Mn-depleted 706 μmol of $DCPIPH_2$ mg chl^{-1} h^{-1}. The residual activities in the absence of $CaCl_2$, NaCl, or DPC, respectively, were less than 10% for Cl^-- and Ca^{2+}-depleted and less than 5% for NH_2OH-washed PSII of the given activities. (B) D1 protein loss. The content of the D1 protein was determined by immunoblotting using a polyclonal antibody. For the determination of the D1 content the standard deviation was approximately ±10%. (C) Immunoblot of photoinhibited PSII-enriched membrane fragments. Samples were illuminated for 10 min, lanes 1−4, and 25 min, lanes 5−8, respectively; 2.8 μg of Chl was used per lane; Active PSII, lanes 1, 5; Cl^--depleted PSII, lanes 2, 6; Ca^{2+} -depleted PSII, lanes 3, 7; Mn-depleted PSII, 4, 8; lane 5 shows needle-marks for the molecular weight markers.

D1 was almost completely lost after 50 min of photoinhibition in Mn-depleted PSII, while little loss of D1 was observed in control and Ca^{2+}- and Cl^--depleted PSII (Figure 1B).

A comparison between the kinetics of PSII activity and D1 loss in these samples indicates that they are not directly correlated. The higher susceptibility of Cl^--depleted PSII compared to Ca^{2+}-depleted PSII is not reflected in an increased D1 loss. Indeed the D1 loss in Ca^{2+}- and Cl^--depleted PSII is the same as in control PSII despite the big differences in photoinhibition of PSII activity. A direct

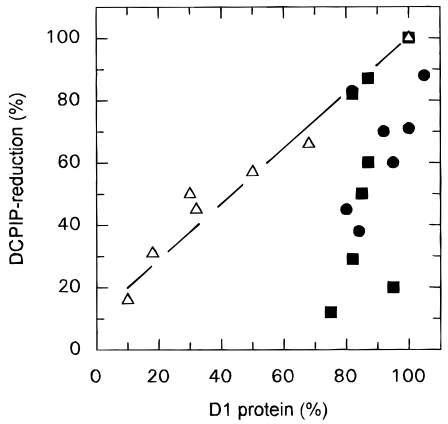

FIGURE 2: Correlation between activity loss and D1 loss during photoinhibition for Cl^--depleted (closed squares), Ca^{2+}-depleted (closed circles), and Mn-depleted (open triangles) PSII-enriched membrane fragments. The same data were used as for Figure 1.

comparison of PS II activity and D1 loss shows this discrepancy more clearly (Figure 2). There is a linear, nearly direct correlation between D1 loss and activity loss in Mn-depleted PSII. In the other samples this is not the case in that they lose their activity while maintaining most of their D1 protein intact during the experiment.

To determine whether these differences reflect distinct mechanisms of photoinhibition, we investigated the occurrence of reactive oxygen species in the above samples during photoinhibition by the use of spin-trapping EPR spectroscopy. Singlet oxygen can be trapped by TEMP and is characteristic to acceptor-side-induced photoinhibition. Figure 3A shows that $^1O_2$ was detected in control PS II membranes which were subjected to photoinhibition for 25 min. In Cl^-- and Mn-depleted PSII membranes no such signal was seen. In Ca^{2+}-depleted PSII membranes a weak signal was observed.

DMPO will trap carbon-centered radicals which are characteristic of acceptor-side-induced photoinhibition and hydroxyl radicals which are characteristic of donor-side-induced photoinhibition, as described earlier (*22*, *23*). Figure 3B shows that 25 min of photoinhibition led to the formation of a carbon-centered radical DMPO adduct in the control. Hydroxyl radical formation dominated in Ca^{2+}, Cl^--, and Mn-depleted PSII membranes (Figure 3B). For clarification, the signals from trapped hydroxyl radicals are shaded in Figure 3B. In Ca^{2+}- and Cl^--depleted PSII membranes, a signal of the carbon-centered radical is also present. This may result in part from a fraction of active centers (approximately 10%, see Materials and Methods) which behave like control membranes.

The EPR results indicate that, when the water-splitting complex is inhibited, photoinhibition results in mainly the formation of hydroxyl radicals, irrespective of the type of donor side inactivation. Thus, the discrepancy between the loss of activity and the D1 loss (Figures 1 and 2) cannot be explained by substantial differences in radical chemistry at least as indicated from spin-trapping experiments. Mn-, Ca^{2+}-, and Cl^--depleted PSII membranes show significantly different kinetics of D1 loss, although the same type of free radical formation dominates their photoinhibition.

The question arises why different types of donor-side-inactivated PSII behave so differently under photoinhibition

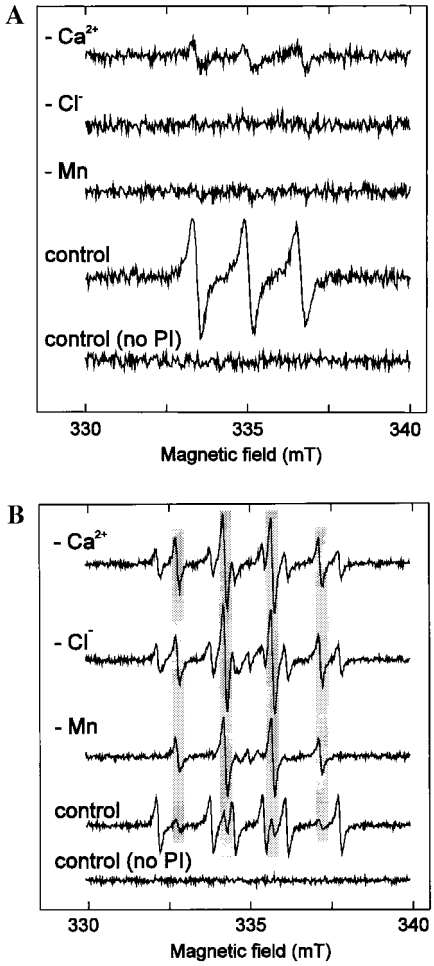

FIGURE 3: Spin trapping of oxygen radicals in Ca^{2+}-, Cl^--, and Mn-depleted and active PSII-enriched membrane fragments which were subjected for 25 min to illumination with white light (1100 μmol of quanta m^{-2} s^{-1}). For clarity the curves have been displaced vertically: (A) singlet oxygen trapping by TEMP; (B) free radical trapping by DMPO.

Table 1: Changes of the Mn Content of PSII Membranes during Photoinhihition Measured by Atomic Absorption Spectroscopy[a]

photoinhibition time (min)	Mn content/220 Chl		
	control	Ca^{2+}-depleted	Cl^--depleted
0	4.3	4.0	3.6
15	4.0	3.9	3.1
30	4.2	3.2	3.7
45	4.3	3.0	3.3
60	4.5	2.9	3.9

[a] The samples (150 μg of Chl/mL) were incubated with white light (1100 μmol of quanta m^{-2} s^{-1}) for the time indicated and were washed twice to remove the released Mn. The control (untreated PSII membranes) contained 4.2 ± 0.3 Mn/PSII reaction center (average from 6 different samples).

with regard to loss of activity and D1 loss. Ca^{2+}-, Cl^--, and Mn-depleted PSII are all inhibited in water oxidation, but their donor sides are different: both Ca^{2+}- and Cl^--depleted PSII contain their Mn cluster. This might imply that Mn release is a prerequisite for D1 loss. To see whether the Mn cluster is maintained during photoinhibition, we measured the Mn content per reaction center in control and Ca^{2+}- and Cl^--depleted PSII by atomic absorption prior to and after photoinhibitory illumination (Table 1). As expected, untreated and Ca^{2+}-depleted PSII membranes con-

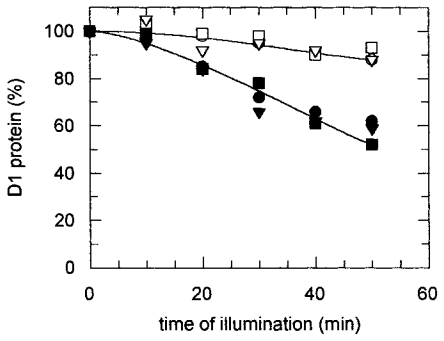

FIGURE 4: D1 protein loss during photoinhibition of Cl^-- (squares) and Ca^{2+}-depleted (triangles) and active (circles) PSII-enriched membrane fragments. The samples were incubated with white light (1100 μmol of quanta m^{-2} s^{-1}) at room temperature. Open symbols: samples were kept for 1 h on ice and 15 min at room temperature after the photoinhibition treatment. Closed symbols: samples were subjected to a NH_2OH treatment (1 h on ice) after the photoinhibition treatment before determination of the D1 content.

tained approximately 4 Mn/220 Chl; in Cl^--depleted PSII the content was a little lower, 3.6 Mn/220 Chl. NH_2OH-washed PSII membranes contained less than 0.5 Mn/220 Chl. During photoinhibition active PSII (control) and Cl^--depleted samples showed practically no Mn release at the light intensity used. Ca^{2+}-depleted PSII seems to lose some Mn but not more than one Mn per reaction center. These measurements are consistent with the idea that D1 was degraded to a large extent only in the absence of Mn.

Figure 4 shows that indeed the Mn content of the samples is important for the D1 loss. In active and Ca^{2+}- and Cl^--depleted PSII, up to a 40% loss of the D1 protein could be observed when the Mn cluster was removed in the dark after the samples had been subjected to photoinhibitory illumination. No difference in the amount of D1 loss could be seen between active and Ca^{2+}- and Cl^--depleted PSII. Practically no D1 loss was observed in samples which contained their Mn cluster, as shown also in Figure 1.

To test in a different way whether the presence of Mn has any effect on photodamage and D1 loss, we measured the effect of photoinhibition on PSII activity and the D1 protein content in photoactivated PSII. The photoactivation procedure was performed either in the presence of both Ca^{2+} and Mn^{2+} or only in the presence of one of these cofactors, either Ca^{2+} or Mn^{2+}. Photoactivation of NH_2OH-treated PSII membranes in the presence of both Ca^{2+} and Mn^{2+} resulted in approximately 30% restoration of oxygen evolution as compared to untreated PSII and the sample contained a large amount of inactive centers (data not shown).

Figure 5 shows the effect of photoinhibition on these photoactivated samples. After photoactivation, samples were washed twice to remove unbound Mn in order to avoid electron donation from free Mn^{2+}, which retards photodamage (*9*, *18*). Without this additional washing, the presence of free Mn^{2+} almost completely suppressed electron transport inactivation by photoinhibition during the first 30 min (data not shown). Before measuring PSII activity, the photoactivated samples and the control were subjected to a NH_2OH treatment. The Mn cluster was thereby removed to ensure the same accessibility for the artificial donor DPC to the different samples. As expected, PSII membranes photoactivated in the presence of Mn^{2+} and Ca^{2+} were less susceptible to photoinhibition than Mn-depleted PSII (Figure 5A). Nevertheless, a faster loss of activity was found in photoactivated PSII compared to active PSII (control); this is attributed to the fact that photoactivation occurs in only a fraction of the centers. Samples subjected to the reactivation procedure in the absence of Ca^{2+} show no oxygen-evolving activity, and their PSII activity was almost as susceptible to photoinhibition as Mn-depleted PSII. Samples subjected to the reactivation procedure in the presence of only Ca^{2+} showed the same extent of photoinhibition as Mn-depleted PSII.

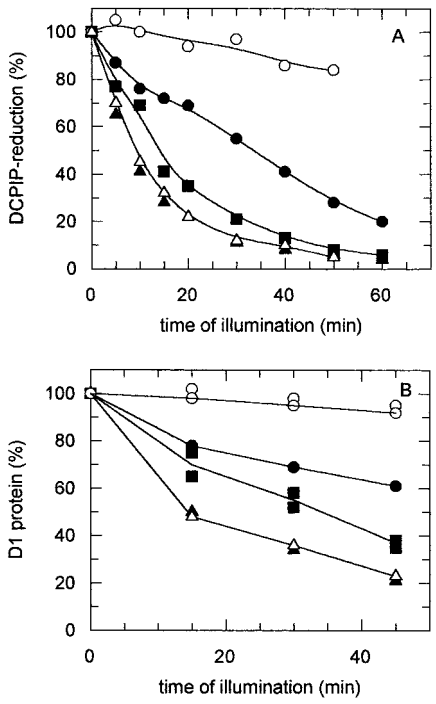

FIGURE 5: Photoinhibition of photoactivated PSII, photoactivated in the presence of Ca^{2+} and Mn^{2+} (closed circles), only in the presence of Mn^{2+} (closed squares), or only in the presence of Ca^{2+} (open triangles), NH_2OH-washed (closed triangles), and active (open circles) PSII-enriched membrane fragments. Photoinhibition treatment and measurements were performed as described in Figure 1. (A) Photoinhibition of PSII activity. All samples were subjected to a NH_2OH treatment before PSII activity was measured in the presence of 1 mM DPC and DCPIP. The activity of the samples was the following: control (active) 650 μmol of $DCPIPH_2$, photoactivated (Ca^{2+}, Mn^{2+}) 613 μmol, photoactivated (Mn^{2+}) 580 μmol, photoactivated (Ca^{2+}) 560 μmol, and NH_2OH-washed 571 μmol of $DCPIPH_2$. (B) D1 protein loss. The content of the D1 protein was determined by immunoblotting.

Figure 5B shows the amount of D1 protein during photoinhibition in photoactivated samples in comparison with untreated and Mn-depleted PSII. The photoactivation procedure performed either in the presence of both Ca^{2+} and Mn^{2+} or in the absence of Ca^{2+} retarded the loss of the D1 protein. The D1 loss was slightly enhanced if the photoactivation procedure was performed in the absence of Ca^{2+} as compared to samples reactivated in the presence of both Ca^{2+} and Mn^{2+} (Figure 5B). This effect might be due to an instability of the Mn cluster in the absence of Ca^{2+}. No protection of the D1 protein against degradation was observed in samples subjected to the reactivation procedure in the presence of only Ca^{2+}. This result indicates that protection against D1 loss is not related to the activity of the water-splitting complex but rather to the binding of Mn.

Table 2: Mn Content of Photoactivated PSII Membranes Measured by Atomic Absorption Spectroscopy

Mn Content of PSII Subjected to the Reactivation Procedure[a]

sample	Mn/220 Chl
untreated PSII membranes	4.2
PSII + 100 μM Mn^{2+} incubated in the dark	4.5
NH_2OH-washed PSII membranes	0.4
NH_2OH-washed, 100 μM Mn^{2+} in the dark	0.7
NH_2OH-washed, 100 μM Mn^{2+} in the light	8.2
NH_2OH-washed, 50 mM $CaCl_2$ + 100 μM Mn^{2+} in the light	2.8

Changes of the Mn Content during Photoinhibition of Photoactivated PSII[b]

photoinhibition time (min)	Mn content/220 Chl: photoactivated with Ca^{2+}, Mn^{2+}	Mn content/220 Chl: photoactivated with Mn^{2+}
0	2.8	8.2
15	2.6	6.5
30	2.4	4.5
45	2.5	4.0
60	2.1	3.2

[a] NH_2OH-washed samples were incubated for 30 min at 30 μmol of quanta m^{-2} s^{-1} with the $MnCl_2$ and $CaCl_2$ concentrations indicated in the table, then washed twice before measuring atomic absorption. [b] The samples (150 μg of Chl/mL) were incubated with white light (1100 μmol of quanta m^{-2} s^{-1}) for the time indicated and were washed twice to remove the released Mn.

Spin-trapping experiments using DMPO revealed more heterogeneous signals in photoactivated samples than observed for either the control or the Mn-depleted PSII. Both hydroxyl and carbon-centered radicals were present. However, in PSII subjected to the reactivation procedure in the presence of Mn and Ca^{2+}, the formation of carbon-centered radicals dominated, while in PSII subjected to the reactivation procedure either in the presence of Mn alone or in the presence of Ca^{2+} alone, mainly hydroxyl radicals were trapped (data not shown). Singlet oxygen production was practically not detectable with TEMP in either of these samples (data not shown).

Upon photoactivation of NH_2OH-washed samples in the presence of Mn^{2+} and Ca^{2+}, 2.8 Mn/220 Chl were ligated as shown in Table 2. As mentioned above, only 30% of the centers were able to evolve oxygen. Presumably, in part of the centers, the normal stable tetramanganese cluster was formed while other centers did not ligate Mn. This situation resulted in an average of 2.8 Mn/220 Chl, a value which has been reported for photoactivated PSII by other groups (e.g., refs *27* and *43*). Photoactivation in the presence of only Mn^{2+} led to a large increase of nonfunctional membrane-bound Mn, 8.1 Mn/220 Chl. These samples were unable to evolve oxygen, and no multiline signal could be detected by EPR (data not shown). It is unlikely that this is only caused by Mn bound to the Ca^{2+} site at the donor side of PSII, because a multiline EPR signal can be detected under this condition (*56*). Addition of Ca^{2+} to this sample resulted in a loss of 4 Mn/220 Chl, the formation of the normal tetramanganese complex, and the partial restoration of the water-splitting activity. In low-temperature EPR the multiline signal and the "hexaquo" signal of free Mn^{2+} could be detected (data not shown). Similar results were reported by Chen et al. (*43*), who observed ligation of up to 18 Mn/200 Chl. Under similar experimental conditions as used here, they also obtained approximately 8 Mn/220 Chl. Incubation of NH_2OH-washed PSII membranes with $MnCl_2$ in the dark did not lead to the ligation of Mn.

As shown in Table 2, photoactivation in the presence of both Ca^{2+} and Mn^{2+} led to a quite stable ligation of Mn, and very little Mn was released even after 60 min of photoinhibition. Illumination of PSII membranes subjected to the reactivation procedure in the absence of Ca^{2+} (8 Mn/220 Chl) led to the loss of Mn^{2+} during the photoinhibition: 5 Mn/220 Chl were lost after 60 min. We assume that some centers lost all ligated Mn while other centers lost four Mn and possessed the normal tetramanganese cluster.

DISCUSSION

A comparison of the loss of PSII electron transport activity and D1 protein in active, Ca^{2+}- and Cl^--depleted, and photoactivated PSII membranes shows that the kinetics of the inhibition of PSII activity do not correlate with the kinetics of the D1 loss: faster functional impairment does not necessarily imply a proportionally faster loss of D1 protein (Figures 1, 2, 5).

There exists not only a discrepancy between activity loss and the extent of D1 loss but also a large difference in the light susceptibility of the PSII activity of Ca^{2+}- and Cl^--depleted PSII (Figure 1A). Both Ca^{2+} and Cl^- depletion lead to comparable lesions on the donor side of PSII (*44*), but Ca^{2+}-depletion causes in addition an upshift of the midpoint redox potential of Q_A/Q_A^- by 150 mV (*45*, *46*). Q_A stays at its normal low potential in Cl^--depleted samples (*34*). The lower susceptibility to light of Ca^{2+}-depleted PSII was interpreted in terms of a model proposed earlier (*32*): the upshift of the E_m of Q_A/Q_A^- in Ca^{2+}-depleted PSII results in protection of the reaction center from photoinhibition by changing the dominant charge recombination pathway to one which does not involve formation of the $P680^+Ph^-$ radical pair and thus lowers the probability of $^3P680$ and subsequent $^1O_2$ formation. In Cl^--depleted PSII, $^1O_2$ formation was expected, because the route of charge recombination within PSII should be similar to that of active PSII (*34*). However, no $^1O_2$ could be detected by spin trapping (Figure 3A). This does not entirely exclude $^1O_2$ generation: failure to detect $^1O_2$ might be explained by production below the sensitivity of the trap or by the presence of a target more attractive than the singlet oxygen trap. However it is also possible that charge recombination is not the dominant reaction responsible for photodamage in Cl^--depleted PSII under the conditions used here (see ref *6*). In inactive PSII, a range of different reactions can take place, and during prolonged photoinhibition, highly oxidizing species such as Tyr_Z^+ and/or Chl^+ accumulate at the donor side and might be responsible for the photoinactivation (e.g., refs *6* and *8*−*11*). After 25 min of high-intensity illumination, hydroxyl radicals were trapped in all samples with an inactive water-splitting complex (Figure 3B). Longer photoinhibitory light treatment resulted in the production of a bigger variety of free radicals, regardless of the intactness of the water-splitting complex prior to illumination. After a longer illumination time, hydroxyl radicals could be trapped also in untreated control samples. Furthermore, it is possible that another radical species, for example, superoxide, is involved in photoinhibition which cannot be detected by the spin traps used here.

Chen et al. (*18*) proposed that superoxide might be involved in the photoinhibition of Mn-depleted PSII.

Our data demonstrate that the extent of D1 protein loss during photoinhibition is affected neither by the water-splitting activity of the sample nor by the chemical nature of the reactive oxygen species generated (Figure 3), and thus appears to be independent of the mechanism of photoinactivation (donor side versus acceptor side route). However, D1 loss appears to depend on the presence or absence of manganese bound to PSII irrespective of the functional state of the enzyme (Figure 4). Experiments using PSII that had been subjected to the reactivation procedure in the presence of Mn only (i.e., without Ca^{2+}) indicate that the rebinding of Mn is sufficient to protect D1 from degradation (Figure 5B).

Tables 1 and 2 show that, during photoinhibition at the given light intensity within our standard deviation (±7%), virtually no Mn is released in active PSII, Ca^{2+}- and Cl^{-}-depleted PSII, and photoactivated PSII. Only PSII membranes subjected to the reactivation in the presence of Mn but without Ca^{2+} released Mn during photoinhibitory illumination. This could be due to the instability of what appears to be an abnormal Mn cluster (i.e., 8 Mn/PSII, see ref *43*). Although no Mn release was measured in active PSII or Ca^{2+}- or Cl^{-}-depleted PSII, 10%−20% of the D1 protein was lost (Figure 1B, 2, 4). This might reflect a certain proportion of PSII centers lacking Mn in all samples.

In the literature, however, Mn release is reported to occur during photoinhibition of untreated thylakoid membranes (*5*), PSII-enriched membranes (*47*), and Cl^{-}-depleted PSII membranes (*48*). However, the experimental conditions were very different from the protocol used here: Virgin et al. (*5*) and van Wijk et al. (*47*) used a much higher light intensity, 7000 and 3500−4000 μmol m^{-2} s^{-1}, respectively, conditions which led to almost complete loss of activity. Our data are not directly comparable to these studies since our study appears to compare with their first experimental points in their time-course studies of D1 loss (or even earlier times if one compares activity loss). Jegerschöld and Styring (*48*) used sulfate treatment to remove chloride which seems to specifically perturb the Mn cluster (*49*) and removes the 17 and 23 kD extrinsic polypeptides (*37*), thus exposing the Mn to reductive attack (*50*). In addition the higher pH used in that study is likely to further destabilize the Mn cluster.

A kinetic difference between the loss of PSII activity and the loss of D1 has already been reported for the photoinhibition of active PSII (e.g., refs *5* and *51*). D1 degradation and Mn release occurred together after the photoinactivation of PSII (*5*). Inhibition of PSII activity and D1 degradation could be separated by their different temperature sensitivities. The D1 protein was not degraded at low temperature (2 °C) while photoinduced loss of PSII activity still occurred (*7*). Van Wijk et al. (*47*) showed that Mn is released during strong illumination at 2 °C and showed that the loss of Mn occurred with kinetics matching those for triggering of the D1 protein for degradation. The present work indicates that Mn release is a prerequisite for D1 loss. In the earlier work the loss of the Mn was seen as being associated with a light-induced conformational change brought about by a reaction mediated by photochemistry in the damaged reaction center. In the present work, it appears rather that the debinding of the Mn may itself be responsible for the conformational change triggering finally the degradation of D1 in photodamaged PSII. In active and Ca^{2+}- and Cl^{-}-depleted PSII, D1 loss could be observed when the samples were first subjected to the photoinhibitory treatment which was then followed by a subsequent removal of the Mn cluster in the dark by NH_2OH treatment (Figure 4).

Photoinhibition of PSII activity and D1 degradation have also been studied previously in donor-side-inactivated PSII (*6*, *51*, *52*). Jegerschöld et al. (*6*) described for Cl^{-}-depleted PSII that the net loss of D1 lags behind the inhibition of electron transport. They reported a faster loss of the D1 protein in Tris-washed PSII membranes than in Cl^{-}-depleted PSII under identical experimental conditions (*51*). Also Spetea et al. (*52*) showed that the D1 loss induced by photoinhibition was faster in Tris-washed than in low-pH-treated (Ca^{2+}-depleted) thylakoid membranes. Neither group pointed out that the destruction of the Mn cluster accelerates the loss of D1 although this effect can be seen in their data.

The data presented here show that Mn binding appears to protect the D1 protein against degradation. Different possibilities can be envisaged to explain this effect: (1) Mn binding is involved in keeping a certain conformation of the protein, and only after a conformational change is the protein susceptible to degradation; (2) the Mn binding inhibits the access to the target for the protease reaction; (3) Mn binding results in structural changes which affect the acceptor side cleavage site (N. B. calcium effects across the membrane, which affect the E_m of Q_A/Q_A^{-}, have been described (*45*, *46*, see also ref *57*); and (4) the presence of Mn might allow protective electron-transfer reactions in inactive PSII, for example, a cyclic electron flow around PSII.

The question arises which process causes Mn loss under conditions of D1 degradation observed under higher intensity illumination reported in the literature (i.e., refs *5*, *6*, *47*, and *53*) and even under normal function for D1 breakdown. One possibility is that, during photoinhibition of photoinactivated PSII reaction centers, reductants such as H_2O_2 are produced which reduce Mn in higher oxidation states to Mn^{2+} which is released from its binding site. It has been reported in the literature that the extrinsic proteins and Mn are released and that D1 is degraded simultaneously (*53*, *54*). We propose that first the extrinsic proteins are released, then reductive loss of Mn occurs, and finally D1 is degraded. It might be possible that even back electron flow in the photoinactivated PSII could reduce higher oxidation states of Mn if the system was perturbed.

From the present results it can be speculated that D1 degradation in vivo occurs after the loss of the Mn cluster. Indeed Mn loss might be the final event before the proteolysis of the D1 protein starts. It has been proposed that, during photoinhibition, active PSII is converted into inactive centers (PSIIβ centers, Q_B-nonreducing centers) which migrate from the grana stacks into the stroma lamellae of the thylakoid membrane. It was proposed that D1 degradation and reassembly of functional PSII might take place in the margins or in the stroma lamellae (PSII damage repair cycle, see refs *2*, *3*, and *33*). It is possible that damaged PSII may maintain the Mn cluster while in the grana stacks and may undergo Mn loss in the margins or in the stromal membranes.

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