Spectroscopic Characterization of Intermediate Steps Involved in Donor-Side-Induced Photoinhibition of Photosystem II[†]

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ABSTRACT: The reaction center protein D1 in photosystem II shows a high turnover during illumination. The degradation of the D1 protein is preceded by photoinhibition of the electron transport in photosystem II. There are two distinct mechanisms for this: acceptor-side- and donor-side-induced photoinhibition. Here, donor-side-induced photoinhibition was studied in photosystem II membranes after Cl^- depletion or washing with tris(hydroxymethyl)aminomethane (Tris) which destroys water oxidation, reversibly or irreversibly, respectively. Photoinhibition after these treatments leads to fast degradation of the D1 protein, and the mechanism behind this was investigated. Illumination of Cl^- depleted photosystem II membranes resulted in a rapid and simultaneous inhibition of Cl^--reconstitutable oxygen evolution, loss of 2 Mn ions per photosystem II center, increase in the electron transfer between the electron donor diphenylcarbazide and electron acceptor 2,6-dichlorophenolindophenol, and an increase in the EPR signal II_{fast} from tyrosine-Z^{ox}. The destruction of the Mn cluster leads to the loss of oxygen evolution and to an increased accessibility for diphenylcarbazide to donate electrons to Tyr-Z^{ox}. The increase in the EPR signal from Tyr-Z^{ox} can be explained by slower reduction kinetics of Tyr-Z^{ox} due to the Mn release. On a longer photoinhibition time scale, a decrease in the amplitude of Tyr-Z^{ox} and inhibition of the electron transport from diphenylcarbazide to 2,6-dichlorophenolindophenol occurred simultaneously in both Cl^--depleted and Tris-washed photosystem II membranes. These slower photoinhibition reactions were then studied in detail in Tris-washed photosystem II membranes. Compared to photoinhibition of Tyr-Z^{ox}, the EPR signal from tyrosine-D^{ox} decreased much slower. Tyr-D^{ox} was photoinhibited in parallel with the EPRsignals from reduced Q_A, reduced pheophytin, and an oxidized chlorophyll radical (chlorophyll$_Z$). This shows that the acceptor side components and the primary charge separation reaction ($P680^+$ pheophytin$^-$) were operational although Tyr-Z was inactivated. The amount of the D1 protein also declined in parallel with Tyr-D^{ox}, which shows that the D1 protein is not damaged until long after the Mn complex and Tyr-Z have become inactivated. Instead, it is likely that the strongly oxidizing $P680^+$ is responsible for the damage to the D1 protein.

Photoinhibition of photosynthesis (Powles, 1984) occurs when photosynthetic organisms are exposed to high light intensities and results in inhibition of the electron transfer through photosystem II (PSII)[1] and subsequent degradation of the D1 reaction center protein (for reviews, see: Barber & Andersson, 1992; Prasíl et al., 1992; Aro et al., 1993; Styring & Jegerschöld, 1994).

PSII is a large enzyme complex in oxygenic photosynthetic organisms (Debus, 1992; Vermaas et al., 1993; Dau, 1994). Two subunits, the D1 and D2 proteins, form a heterodimer that binds the primary electron donor P680, the intermediary electron acceptor pheophytin, the first and second quinone acceptors, Q_A and Q_B, and the Mn cluster involved in the oxidation of water. Also present are two redox-active tyrosine residues, Tyr-Z and Tyr-D, on the D1 and D2 proteins, respectively. Tyr-Z is the redox interface between the manganese cluster and P680 (Babcock et al., 1989) and has recently been proposed to participate in water oxidation through the abstraction of a hydrogen atom from water (Gilchrist et al., 1995; Hoganson et al., 1995). Tyr-Z is oxidized in nanoseconds after the charge separation and is then rapidly reduced from the manganese cluster; in the absence of the manganese cluster, Tyr-Z functions more slowly (Babcock & Sauer, 1975). Tyr-D is an accessory electron donor in PSII which is stable for hours in the dark (Babcock & Sauer, 1973). Both tyrosines give rise to EPR spectra that are quite similar in shape but have very different decay kinetics; the spectrum from Tyr-Z^{ox} is denoted signal II_{fast} or signal $II_{very\ fast}$ (depending on the intactness of the water oxidizing complex) while the spectrum from Tyr-D^{ox} is known as signal II_{slow} (for a review on EPR spectroscopy in PSII, see: Miller & Brudvig, 1991).

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[1] Abbreviations: Chl, Chlorophyll; Chl$_Z$, accessory chlorophyll electron donor in PSII; DCPIP, 2,6-dichlorophenolindophenol; DPC, diphenylcarbazide; EDTA, ethylenediaminotetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid); PPBQ, phenyl-*p*-benzoquinone; PSII, photosystem II; P680, primary electron donor in PSII; Q_A, Q_B, first and second quinone electron acceptors in PSII; Tris, tris(hydroxymethyl)aminomethane; Tyr-D, tyrosine-161 on the D2 protein; accessory electron donor in PSII; Tyr-Z, tyrosine-161 on the D1 protein; immediate electron donor to $P680^+$.

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Two distinct mechanisms may lead to photoinhibition of PSII; acceptor-side-induced and donor-side-induced photoinhibition (Styring et al., 1990a; Barber & Andersson, 1992; Styring & Jegerschöld, 1994; Telfer & Barber, 1994; Hideg et al., 1994; Ohad et al., 1994). Acceptor side photoinhibition involves sequential reduction and protonation of Q_A, finally leading to PSII centers without functional Q_A (Styring et al., 1990b; Setlík et al., 1990; Nedbal et al., 1990; Andersson et al., 1992; Vass et al., 1992a; Styring & Jegerschöld, 1994). PSII centers in this state are incapable of secondary electron transfer although the primary charge separation still remains functional. Charge separation in the absence of a functional acceptor side leads to recombination reactions which promote formation of chlorophyll triplets at P680 (Mathis et al., 1989; van Mieghem, et al., 1989; Vass et al., 1992a; Vass & Styring, 1993). ^{3}P680, in turn, cross reacts efficiently with molecular oxygen to form singlet oxygen which damages the D1 protein, thereby triggering its degradation (Mathis et al., 1989; Vass et al., 1992a; Macpherson et al., 1993; Styring & Jegerschöld, 1994; Telfer & Barber, 1994; Hideg et al., 1994; Keren et al., 1995).

Damage to the D1 protein may also be caused by the highly oxidizing components on the donor side of PSII. P680, Tyr-Z, and the higher oxidation states of the water oxidizing complex reach potentials of +800−1100 mV, and the most oxidizing radicals, $P680^+$ and $Tyr\text{-}Z^{ox}$, are potentially dangerous for their surroundings (Styring et al., 1990a; Barber & Andersson, 1992; Styring & Jegerschöld, 1994; Telfer & Barber, 1994). When, for one reason or another, the water oxidizing complex is unable to rapidly deliver electrons, both $Tyr\text{-}Z^{ox}$ and $P680^+$ become abnormally long-lived and may accumulate. This is a dangerous situation, and when the illumination continues, any remaining electron transfer through PSII is rapidly inactivated. This inhibition is termed donor-side-induced photoinhibition and has been studied in various PSII preparations with lesions on the oxidizing side of PSII (Callahan et al., 1986; Theg et al., 1986; Klimov et al., 1990; Blubaugh & Cheniae, 1990; Blubaugh et al., 1991; Jegerschöld et al., 1990; Jegerschöld & Styring, 1991; Chen et al., 1992, 1995), in reaction center preparations (see Telfer & Barber, 1994, and refs therein) and in mutants where the water oxidizing complex is affected (Mayes et al., 1991; Vass et al., 1992b; Rova et al., 1994; Gong & Ohad, 1991, 1995; Keren et al., 1995). The electron transfer is photoinhibited in a sequential series of reactions. Ultimately, the D1 protein is degraded in oxygen-independent reactions that are distinct from the acceptor side photoinhibition reactions (Jegerschöld & Styring, 1991; Shipton & Barber, 1991). Seemingly, the initial target in photoinhibition is Tyr-Z which is inactivated in a reaction that might involve superoxide or hydroxyl radicals (Chen et al., 1992, 1995). In subsequent reactions, the remaining photochemistry, including P680 and Tyr-D, is inhibited. It has also been suggested that one of the first steps in photoinhibition is the irreversible oxidation of a carotenoid (Klimov et al., 1990; Blubaugh et al., 1991; Telfer et al., 1991; De Las Rivas et al., 1993). In several of these mechanistic studies, the degradation of the D1 protein was not measured; hence the coupling of the inhibitory reactions with the protein degradative reactions was not studied.

An important reaction in photosynthesis is the continuous high turnover of the D1 reaction center protein (Barber & Andersson, 1992; Prasíl et al., 1992; Aro et al., 1993; Ohad et al., 1994). The turnover is increased at high light intensities but occurs also at low and moderate light. Therefore, a key question is to what extent acceptor-side and donor-side photoinhibition are relevant *in vivo* and which mechanism is responsible for the extensive turnover of the D1 protein under various conditions. For example, it was recently suggested that Ca^{2+} might be lost from the donor side of PSII due to the acidification of the lumen in high light (Krieger & Weis, 1993; Krieger et al., 1993). This would induce a situation that could lead to donor-side-induced photoinhibition of PSII and enhanced degradation of the D1 protein. Thus, conditions leading to donor-side photoinhibition might occur *in vivo*. Therefore, further molecular studies of donor-side photoinhibition, in order to couple the mechanism leading to inhibition of the electron transfer with the reactions that damage the D1 protein, are needed.

In this paper, we have used EPR spectroscopy, electron transfer assays, and protein analysis in an attempt to further resolve some of the steps that are involved in donor-side photoinhibition. From our experiments, it seems clear that the D1 protein is not damaged until long after the Mn complex and Tyr-Z have become inactivated. Instead, it is likely that $P680^+$ is the species ultimately responsible for the damage to the D1 protein.

MATERIALS AND METHODS

PSII Preparations. PSII enriched membranes were prepared from spinach thylakoids (Berthold et al., 1981) and stored in 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 20 mM Mes, pH 6.3. Depletion of chloride from the PSII enriched membranes was achieved by replacing the chloride with SO_4^{2-} at elevated pH (Ono et al., 1986; Jegerschöld et al., 1990). The PSII enriched membranes were washed twice in 0.4 M sucrose, 1 mM NaCl, 5 mM Na_2SO_4, and 40 mM Mes-NaOH, pH 6.5, and then diluted (to 250 μg of Chl mL^{-1}) with 0.4 M sucrose, 50 mM Na_2SO_4, and 40 mM Hepes-NaOH, pH 7.5, and incubated for 15 min in darkness. The membranes were collected by centrifugation and suspended in 0.4 M sucrose, 50 mM Na_2SO_4, and 40 mM Hepes-NaOH, pH 7.0. After this treatment, the remaining oxygen evolution of the PSII membranes was about 20% of the nontreated control. It could be restored to about 90% of the original activity by the addition of 40 mM NaCl. The oxygen evolving complex was removed from PSII by incubation with 0.8 M Tris-HCl buffer at pH 8.3 in room light for 20 min on ice. The Tris buffer was removed by repeated centrifugation and suspension in a buffer containing 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 20 mM Mes-NaOH, pH 6.3. All preparations were frozen in liquid nitrogen and stored at −80 °C until use.

Photoinhibition Experiments. Photoinhibition of the Cl^--depleted PSII membranes (1.5 mL at 1 mg of Chl/mL) was done by illumination with heat-filtered white light at 1000 $\mu E\ m^{-2}\ s^{-1}$ under continuous stirring in a thermostated cell at 20 °C in 0.4 M sucrose, 50 mM Na_2SO_4, and 40 mM Hepes-NaOH, pH 7.5. Samples for activity measurements and Mn analysis were taken during the photoinhibition treatment, but more than 90% of the initial volume always remained after the completion of the experiment. Cl^--depleted PSII membranes are not stable for extended periods of times even in the dark. Therefore, each photoinhibition

experiment was performed immediately after thawing an aliquot of the preparation.

Tris-washed PSII membranes were photoinhibited in a 400 μL cuvette (1 mm thick) at a concentration of 2 mg of Chl/mL in a medium containing 0.4 M sucrose, 25 mM NaCl, 5 mM $MgCl_2$, 40 mM Mes-NaOH, pH 6.5, 2 mM EDTA, and a redox buffer of 15 mM ferri- and 5 mM ferrocyanide. The sample was illuminated with 2000 μE m^{-2} s^{-1} heat-filtered white light at 20 °C. This procedure resulted in a photoinhibited sample which was then further treated to produce one EPR sample for measurements at room temperature or liquid He temperatures. The Tris-washed samples were never kept longer than 1 h on ice prior to the experiments. Individual dark controls were made for every new experimental series and every batch of membranes. The control (100%) value was measured in a non-photoinhibited sample that was treated as a photoinhibited sample except for the illumination period.

Electron Transport Measurements. The light-dependent electron transfer from water or the exogenous electron donor DPC to the electron acceptor DCPIP was measured as DCPIP reduction at 590−540 nm in a Shimadzu UV3000 spectrophotometer equipped with sideways illumination (Jegerschöld et al., 1990). The assay medium consisted of 0.1 M sucrose, 40 mM NaCl, 30 mM HEPES-NaOH, pH 7.1, and 35 μM DCPIP as electron acceptor. The chlorophyll concentration was 5 μg of Chl/mL, and DPC was added to a final concentration of 1 mM from a fresh 30 mM stock solution in methanol.

Protein and Manganese Analysis. The amount of the D1 protein was quantified by Western blotting and ^{125}I-labeling as described before (Virgin et al., 1988; Jegerschöld et al., 1990). Atomic absorption measurements of Mn bound to PSII was done after centrifugation of the samples to remove unbound Mn followed by acidification of the samples (van Wijk et al., 1992) using a Varian Techtron atomic absorption spectrometer equipped with a graphite oven.

EPR Spectroscopy. X-band EPR spectra were recorded with a Bruker ESP300 spectrometer equipped with an Oxford Instruments cryostat and temperature controller for measurements at liquid helium temperatures. The data were processed using the ESP300 software.

For room temperature EPR measurements, the sample was, immediately after the photoinhibition treatment (see above), transferred to the flatcell and the EPR spectra were recorded. For complete oxidation of Tyr-Z (induction of signal II_{fast}) and Tyr-D (SII_{slow}), saturating, heat-filtered white light (1000 W projector) was directed into the cavity with a light guide. Due to the rather fast decay also of Tyr-D^{ox} in both Cl^--depleted and Tris-washed PSII membranes (in the 10−30 min time range), its amplitude was measured after illumination of the sample in the cavity. Tyr-Z^{ox} was measured during continuous illumination of the sample in the cavity. The amplitude of Tyr-Z^{ox} was estimated from the light minus dark difference spectra. For the EPR measurements of the tyrosine radicals in Cl^--depleted PSII membranes, 1 mM EDTA and 0.5 mM PPBQ were added.

Samples from photoinhibited and control Tris-washed PSII membranes intended for EPR measurements at liquid He temperature were centrifuged and resuspended twice to remove the ferri/ferrocyanide. The final suspension was done to a concentration of about 2 mg of Chl/mL in the photoinhibition buffer without ferri/ferrocyanide. Sodium formate at pH 6.5 was added to a final concentration of 25 mM, and the sample was incubated on ice for 20 min in the dark. Formate was added to enhance the size of the Q_A^--Fe^{2+} signal in the EPR measurements that follow (Vermaas & Rutherford, 1984; Styring et al., 1990b). The samples were then transferred to calibrated quartz EPR tubes, illuminated for 2 min in room light to oxidize Tyr-D, frozen to 77 K, and stored in liquid nitrogen. The size of Tyr-D^{ox} in the non-photoinhibited controls represents 1 radical/PSII (Miller & Brudvig, 1991) and is used as an internal standard to which the radical EPR signals from Tyr-Z^{ox}, reduced pheopytin, and oxidized chlorophyll are correlated (Styring et al., 1990b).

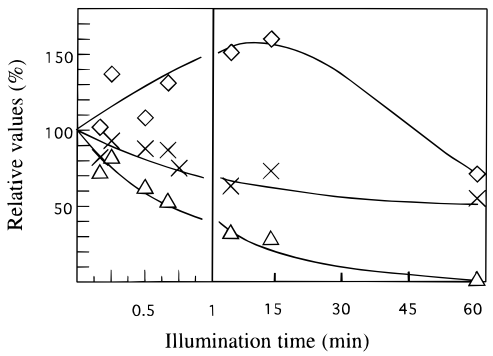

FIGURE 1: Time course for the photoinhibition of Cl^--depleted PSII membranes. The figure shows the inhibition of the reconstitutable electron transport from H_2O to DCPIP (triangles) assayed in the presence of chloride (40 mM) and the electron transport from DPC to DCPIP (diamonds). The remaining membrane-bound Mn (crosses) after photoinhibition was measured after centrifugation of the samples to remove unbound Mn. Note the different time scales: left, 0−1 min photoinhibition; right, 1−60 min photoinhibition.

RESULTS AND DISCUSSION

Early Events during Photoinhibition of Cl^--Depleted PSII Membranes. Removal of chloride from PSII reversibly inhibits the oxygen evolving complex; when saturating chloride is added back (40 mM), about 90% of the control level of the oxygen evolution is reached. Cl^- depletion renders the oxygen evolving capacity extremely sensitive to illumination probably due to an initial light damage at the level of the Mn cluster (Theg et al., 1986; Jegerschöld et al., 1990). Here, these early photoinhibition reactions were studied in further detail. During photoinhibition of Cl^--depleted PSII membranes, the reconstitutable oxygen evolution was rapidly lost (Figure 1). In 60% of the centers, the activity was inhibited with an inhibition halftime of about 1 min while the oxygen evolution in the remaining centers was inhibited with a inhibition halftime of 15−20 min which is still fast compared to the photoinhibition halftime in control membranes (not shown). The biphasic inhibition might reflect the inhomogeneous protein content in PSII induced by the chloride-depletion procedure where high pH and SO_4^{2-} are used to replace Cl^-. Protein analysis revealed that our preparations of chloride-depleted PSII membranes contained more than 95% of the 33 kDa protein while 60−65% of the 23 and 16 kDa extrinsic proteins had been lost (not shown). It is likely that the fast inhibition occurs in centers lacking the 23 and 16 kDa proteins while the more intact centers are more stable (Rova et al., 1994).

The electron transfer from DPC to DCPIP (i.e., between Tyr-Z and the Q_B site) was also measured during photoin-

hibition (Figure 1). Interestingly, the capacity for electron transfer from DPC to DCPIP increased during the initial phase of the photoinhibition to reach a maximal value of about 150% of the starting value (Figure 1). Interestingly, the maximal level was similar to the rate observed in Tris-washed membranes prior to photoinhibiton. The halftime for the increase correlates well with the halftime for the inhibition of the oxygen evolution. This indicates that the donation site for DPC, which is probably close to Tyr-Z or Tyr-Z itself, becomes more accessible for donation after the Mn cluster has been inactivated. Subsequently, on a slower time scale, the electron transfer from DPC to DCPIP was also inhibited.

This result is seemingly in conflict with our earlier results where no increase, but only a slow inhibition of the DPC-mediated electron transfer was observed during photoinhibition of Cl^--depleted PSII membranes (Jegerschöld et al., 1990). However, this can be explained by differences in the measuring protocols. Our earlier measurements were performed after removal of the Mn cluster by Tris-washing of the photoinhibited centers (as well as in the non-photoinhibited control samples). Tris-washing is known to facilitate electron donation from DPC, probably by the removal of the extrinsic subunits and the Mn cluster (Hsu et al., 1987; Preston & Seibert, 1991). Contrary to this, the measurements in Figure 1 were performed directly after photoinhibition. Therefore, a possible explanation for the observed increase in the DPC donation is that the photoinhibition results in release of manganese from the water oxidizing complex. This would also explain that the inhibition of the oxygen evolution and the increase in the DPC donation occurred simultaneously. This effect would have been overlooked in the earlier experiments since the Mn cluster was removed by Tris-washing prior to the electron transfer measurements (Jegerschöld et al., 1990).

The hypothesis that the Mn cluster was destroyed during photoinhibition was tested by measuring the Mn content in the membranes. There were 4 Mn ions/220 Chl in the intact PSII enriched membranes (not shown). The Cl^--depletion procedure induces some loss of manganese, and in our non-photoinhibited Cl^--depleted PSII membranes there are 3.7−3.8 Mn ions/220 Chl (not shown). Illumination, in the absence of chloride, leads to loss of manganese from the membranes (Figure 1). Approximately 30% of the manganese ions are lost in correlation with the rapid inhibition of the oxygen evolution, and another 20% of the manganese ions are lost together with the slow inhibition of the oxygen evolution (Figure 1). The rest of the manganese (about 50%) remained bound to the membranes for a considerable time after complete inhibition of the oxygen evolution and is probably not released until the D1 protein has become degraded (Virgin et al., 1988).

It is not entirely clear how many manganese ions are lost from each PSII center during the fast manganese release (Figure 1). However, since approximately 30% of the manganese was lost together with inhibition of 60% of the oxygen evolution, it seems likely that two Mn ions are lost from each inactivated PSII center. This would also be in agreement with the 50% of the manganese remaining after complete inhibition of oxygen evolution (Figure 1). Thus, these experiments indicate that, in centers lacking chloride, two of the Mn ions in the Mn cluster are very light-sensitive and easily released to the medium as a consequence of photoinhibition.

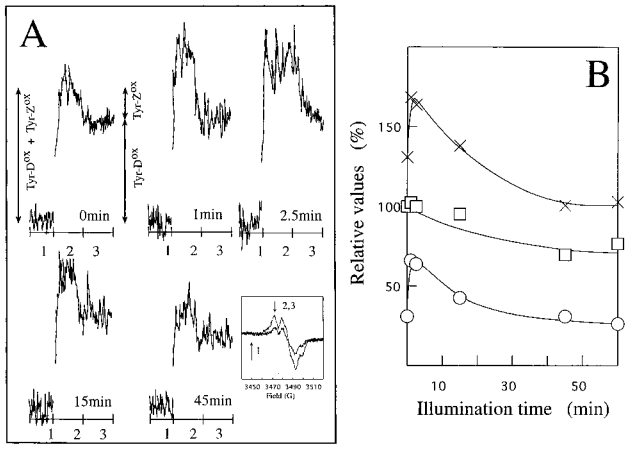

FIGURE 2: Effects of illumination of Cl^--depleted PSII membranes. (A) The effects of photoinhibition on Tyr-Z^{ox} and Tyr-D^{ox} measured by room temperature EPR spectroscopy. Each trace represents one sample that was illuminated, prior to the EPR measurements, for a time period indicated under the trace. For each trace, a baseline was recorded (part 1) at 3450 G outside the Tyr-Z EPR spectrum (marked by arrow 1 in the inset). Parts 2 and 3 in each trace were recorded at the low field shoulder of the tyrosine EPR spectrum (marked by arrow 2,3 in the inset). Part 2 was recorded during continuous illumination to induce Tyr-Z^{ox} and reflects both tyrosine radicals. Part 3 reflects Tyr-D^{ox} only and was recorded in the dark immediately after the illumination. The difference between part 2 and part 3 reflects Tyr-Z^{ox} alone. 40 mM Cl^-, 1 mM EDTA, and 0.5 mM PPBQ were present during the EPR measurements except that Cl^- was omitted in the non-photoinhibited sample ($t = 0$ min). The total illumination time was 5 ± 1 s. The inset shows representative EPR spectra from a Tris-washed sample. The inner trace was recorded in the dark and originates from Tyr-D^{ox}. The outer trace was recorded during illumination. The difference between this and the dark-recorded spectrum represents Tyr-Z^{ox}. (B) Time course for the effects of photoinhibition on the induction of: (crosses) the total amount of oxidized tyrosine radicals, Tyr-D^{ox} and Tyr-Z^{ox}, represented by part 2 in (A); (open squares) Tyr-D^{ox} alone, represented by part 3 in A and (open circles) Tyr-Z^{ox}, represented by the difference between part 2 and part 3 in (A). EPR conditions: temperature 295 K; microwave frequency 9.77 GHz; microwave power 3 mW; modulation amplitude 3.6 G.

Mn binds more firmly to proteins in more oxidized states, and its release from PSII is facilitated by reductants (Ghanotakis et al., 1984). Thus, the release of Mn from Cl^--depleted PSII membranes might be caused by light-dependent formation of a reductant. One possible reducing agent is H_2O_2 that can be formed on the donor side in PSII upon illumination of Cl^--depleted PSII (Schröder & Åkerlund, 1986; Fine & Frasch, 1992). In fact, it has been shown that the presence of catalase during illumination of Cl^--depleted thylakoids protected against photoinhibition (Bradley et al., 1991). Therefore, we propose that H_2O_2, produced near the Mn cluster during photoinhibition of the Cl^--depleted PSII membranes, induces the release of two Mn^{2+} ions per PSII center.

Sensitivity of the Tyrosine Radicals in PSII to Illumination. In hydroxylamine treated PSII membranes, Tyr-Z and Tyr-D are sensitive to photoinhibition (Blubaugh et al., 1991), with Tyr-Z being more light-sensitive than Tyr-D. We have investigated the light sensitivity of these radicals in chloride-depleted and in Tris-washed PSII membranes in order to correlate their photoinhibition with the fate of other electron transfer components on both the donor and the acceptor sides of PSII.

The effects of photoinhibition on the tyrosine radicals in Cl^--depleted PSII membranes are shown in Figure 2. The

EPR traces shown represent samples that were photoinhibited for the times indicated below the traces. In each trace in Figure 2A, the difference between part 1 and part 3 reflects the amplitude of the EPR signal from Tyr-D^{ox} (recorded in the dark), whereas the difference between part 2 and part 3 reflects the amplitude of the EPR signal from Tyr-Z^{ox} (part 2 was recorded during continuous illumination). It was necessary to keep the illumination period for the measurement of Tyr-Z^{ox} very short to prevent further photoinhibition of the sample in the cavity.

Before photoinhibition of the Cl^--depleted PSII membranes, the EPR signal from Tyr-Z^{ox} (signal II_{fast}) was observed in 31 ± 7% of the PSII centers measured in the absence of Cl^- (related to the EPR signal from Tyr-D^{ox}) (Figure 2A, trace 0 min). This is similar to earlier reports in which about 30% of Tyr-Z^{ox} could be observed by flash illumination in the absence of Cl^- (Boussac et al., 1992). However, during the first minutes of photoinhibition, there is an increase in the EPR signal from Tyr-Z^{ox} (Figure 2A, traces 1 and 2.5 min) measured in the presence of EDTA and Cl^-. After 1 min photoinhibition, the light-induced amplitude of Tyr-Z^{ox} reaches 68 ± 7% of the dark-recorded amplitude reflecting Tyr-D^{ox}. (It should be noted that the latter is not affected during this time of photoinhibition.) It seems as if Tyr-Z in these samples has acquired millisecond reduction kinetics comparable to the situation in Tris-washed membranes (Dau, 1994), indicating that the environment around Tyr-Z in the Cl^--depleted PSII membranes rapidly changes during illumination.

The time course for the effects of light on the EPR signals from the tyrosines in Cl^--depleted PSII membranes is shown in Figure 2B. The initial, fast increase in the amount of observable Tyr-Z^{ox} (Figure 2A) seems correlated to the results in Figure 1, where the electron transfer from DPC to DCPIP initially increased rapidly. Furthermore, this coincided with the loss of 2Mn/PSII (Figure 1). Therefore, we propose that the loss of 2Mn per PSII center slows down the reduction kinetics of Tyr-Z, allowing DPC to compete more efficiently for a donation site. At the same time, the slow reduction kinetics of Tyr-Z allows its detection by EPR spectroscopy during continuous illumination. Interestingly, the observation of the increase in Tyr-Z^{ox} depends on the presence of EDTA during the EPR measurement. In the absence of EDTA, the large increase in EPR signal from Tyr-Z^{ox} was not observed (not shown). In this case, it is possible that the released Mn can function as an electron donor to Tyr-Z, perhaps to the same site to which DPC donates.

On a slower time scale, photoinhibition leads to inactivation of Tyr-Z (Figure 2) and DPC donation (Figure 1). This is probably related to the increased lifetime of Tyr-Z that makes the tyrosine side chain or its surroundings more susceptible to illumination damage. It should also be noted that the EPR signal from Tyr-D^{ox} remained high during the experiment (Figure 2). After 1 h illumination, 70−75% of Tyr-D^{ox} was still observed, while Tyr-Z^{ox} was only observable in 25−30% of the centers.

In order to further resolve the kinetics for photoinhibition of the tyrosines, we turned to Tris-washed PSII membranes in the photoinhibition experiments. From the start, the amplitudes of Tyr-D^{ox} and Tyr-Z^{ox} were identical (Figure 3A). Thereafter, photoinhibition resulted in progressively diminished amplitudes of both tyrosine radicals (Figure 3A).

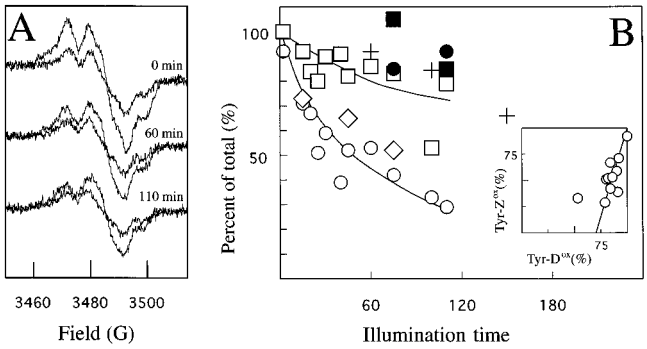

FIGURE 3: Effects of illumination of Tris-washed PSII membranes. (A) The effects of photoinhibition on the radical EPR spectra from Tyr-Z^{ox} and Tyr-D^{ox}. In each pair of spectra, the inner spectrum was recorded in the dark and originates from Tyr-D^{ox}. The outer spectrum was recorded during continuous illumination. The difference between this and the dark-recorded spectrum represents Tyr-Z^{ox}. (B) Time course for photoinhibition of Tyr-Z^{ox} (open circles), Tyr-D^{ox} (open squares), and the DPC-mediated electron transport through PSII (open diamonds). Also shown is the degradation of the D1 protein (crosses). Dark controls are represented by filled circles for Tyr-Z^{ox} and filled squares for Tyr-D^{ox}. The inset in (B) shows the inactivation of Tyr-Z^{ox} versus the loss of Tyr-D^{ox}. EPR conditions: temperature 295 K; microwave frequency 9.77 GHz; microwave power 1 mW; modulation amplitude 3.2 G.

The amplitude of Tyr-D^{ox} decreased slowly during photoinhibition. After 60 and 110 min illumination, 80% and 75%, respectively, of the signal remained. The light-induced signal from Tyr-Z^{ox} was inhibited faster: after 60 min illumination only 40% of Tyr-Z could be photooxidized, while 30% of Tyr-Z remained oxidizable after 110 min illumination (Figure 3A). Thus, electron transfer involving Tyr-Z is clearly more sensitive to illumination than Tyr-D. A comparison of the time curves reveals that photoinhibition of Tyr-Z occurs approximately 5 times faster than photoinhibition of Tyr-D (Figure 3B). The correlation plot (Figure 3B, inset) shows that, after prolonged photoinhibition, as much as 70% of Tyr-D might remain although very little Tyr-Z can be oxidized. It should also be noted that both tyrosines remained oxidizable after prolonged periods in the dark (Figure 3B). This inhibitory pattern in Tris-washed PSII membranes seems to be different from the situation in Cl^--depleted membranes where an initial increase in Tyr-Z^{ox} was observed during photoinhibition (Figure 2). However, in Tris-washed PSII membranes, all PSII centers lack the Mn cluster. Thus, Tyr-Z^{ox} can be detected in all centers prior to photoinhibition (Babcock & Sauer, 1975).

One explanation to the light sensitivity of Tyr-Z is that the illumination somehow modifies the tyrosine or its immediate surroundings on the D1 protein, thus blocking the electron transfer from Tyr-Z to $P680^+$. If so, it is likely that also the Tyr-Z-mediated electron transfer from DPC to DCPIP was inhibited simultaneously. This was indeed the case; the decrease in the DPC-supported electron transport to DCPIP follows the kinetics for the inactivation of Tyr-Z (Figure 3B).

Our results are comparable to those obtained during photoinhibition of NH_2OH/EDTA-washed PSII membranes where Tyr-Z is inactivated first, leaving Tyr-D essentially unaffected (Blubaugh et al., 1991; Chen et al., 1992). These authors found that, during photoinhibition, the reduction of $P680^+$ from Tyr-Z was lost in parallel with the capability to photoaccumulate Tyr-Z^{ox} and to photooxidize added Mn^{2+}. Furthermore, it was concluded that Tyr-Z was inactivated

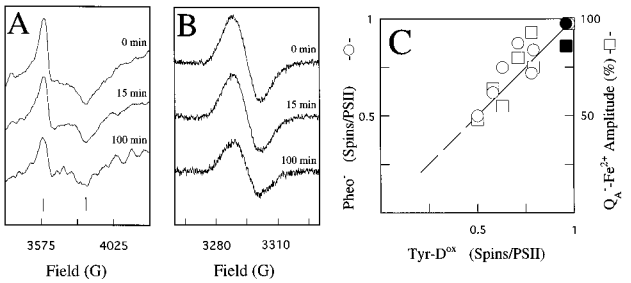

FIGURE 4: Photoinhibition of Q_A and the charge separation reaction in Tris-washed PSII membranes. (A) Decrease of the EPR signal from Q_A^- (the Q_A^--Fe^{2+} signal) during photoinhibition. Q_A was chemically reduced by dithionite (50 mM) in the presence of formate. The bars indicate the peak at $g = 1.82$ and the trough at $g = 1.67$ in the Q_A^--Fe^{2+} signal. EPR conditions: T 4 K; microwave frequency 9.23 GHz; microwave power 32 mW; modulation amplitude 31.4 G. (B) The effect of photoinhibition on the formation of the radical signal from pheophytin$^-$. The reduced samples from (A) were illuminated at room temperature to reduce the pheophytin. EPR conditions: T 15 K; microwave frequency 9.23 GHz; microwave power 63 nW; modulation amplitude 2.8 G. (C) Correlation between the loss of Tyr-D^{ox} and the decrease in the Q_A^--Fe^{2+} EPR signal (open squares) and the decrease in formation of $Pheo^-$ (open circles) as a consequence of photoinhibition of Tris-washed PSII membranes. Controls, kept 100 min in the dark, are represented by filled squares for Q_A^--Fe^{2+} and filled circles for $Pheo^-$.

in an oxygen-dependent fast reaction, possibly between O_2^- and cationic amino acid radicals like Tyr-Z^{ox} or a histidyl side chain, causing their peroxidation or crosslinking (Chen et al., 1992, 1995). Later, these events are followed by damage to the D1 protein in oxygen-independent reactions in the absence of a functional water oxidizing complex (Jegerschöld & Styring 1991; Shipton & Barber, 1991).

Degradation of the D1 Protein. Tyr-Z is situated on the D1 protein. Thus a protein modification at, or in the vicinity of, Tyr-Z might be the triggering event for the degradation of the D1 protein. Therefore, we followed the degradation of the D1 protein by quantitative western blotting (Figure 3B). Despite the fast inactivation of Tyr-Z, the D1 protein remained in the PSII complex for a considerable time. Instead, it was degraded with kinetics that were somewhat slower than the inactivation of Tyr-D. This result clearly indicates that another light-induced component than Tyr-Z is responsible for the damage to the D1 protein.

Effects of Illumination on Electron Transfer Components in PSII other than Tyr-D and Tyr-Z. During acceptor-side photoinhibition, Q_A is sequentially double reduced and protonated, which promotes formation of triplet chlorophyll at P680 (^{3}P680) (Vass et al., 1992a; Vass & Styring, 1993). One important consequence of this mechanism is that the function of Q_A is lost before the primary radical pair formation is inhibited (Styring et al., 1990b; Setlik et al., 1990; Nedbal et al., 1990). The D1 protein is subsequently damaged through interaction with singlet oxygen (Vass et al., 1992a; Hideg et al., 1994).

Here, we tested the order of the inhibitory reactions when Tris-washed PSII membranes were photoinhibited. Figure 4A shows the size of the EPR signal from Q_A^-, the so called Q_A^--Fe^{2+} signal (Miller & Brudvig, 1991), after various times of photoinhibition. A comparison of the signal size in the control and after 15 and 100 min photoinhibition (Figure 4A) shows that the loss of the amplitude occurs with approximately similar kinetics as the inhibition of Tyr-D (Figure 4C). Thus, both functional Q_A and the non-heme iron (both necessary for the formation of the EPR signal) were present and remained stable for a considerable time also after the inactivation of Tyr-Z.

In the same samples that were used for the Q_A^--Fe^{2+} measurements (Figure 4A), the EPR signal from the reduced pheophytin radical was measured after strong illumination of the sample under reducing conditions. Illumination of PSII under these conditions results eventually in quantitative reduction of pheophytin in all centers capable of performing the primary charge separation (Rutherford & Zimmermann, 1984; Styring et al., 1990b; Miller & Brudvig, 1991). The size of the $Pheo^-$ radical EPR signal can therefore be used as a measure of the number of centers in which the charge separation between P680 and pheophytin is still operational. Figure 4B shows typical EPR spectra from $Pheo^-$ ($g \approx 2.0035$; linewidth 11−12 G). The size of the spectrum in the non-photoinhibited control amounted to 1.0−1.1 radical per PSII reaction center as compared to the EPR spectrum from Tyr-D^{ox} that we use as an internal standard of the PSII concentration. During photoinhibition, the amplitude of the $Pheo^-$ signal decreased slowly; after 15 and 100 min photoinhibition, about 90% and 65%, respectively of the amplitude remained, implying that the charge separation was still functional in most centers also after prolonged photoinhibition. In control membranes, the amplitudes of both the Q_A^--Fe^{2+} EPR signal and the $Pheo^-$ radical remained essentially unchanged during 100 min incubation in the dark at room temperature (Figure 4C).

The results from experiments using varying photoinhibition times are plotted in Figure 4C. It is clear that photoinhibition results in simultaneous decrease of both the Q_A^--Fe^{2+} EPR signal, the $Pheo^-$ radical (i.e., the primary charge separation), and Tyr-D. From the room temperature EPR measurements (Figure 3), it was concluded that Tyr-Z was inactivated much faster than Tyr-D. Combination of these data then shows that, despite the fast inactivation of Tyr-Z, the rest of the photochemistry in PSII may remain functional for a considerable time. In subsequent, probably correlated, reactions the primary charge separation, Tyr-D oxidation, and Q_A^- reduction were inhibited. Simultaneously, or with slightly slower kinetics, the D1 protein was degraded (Figure 3).

Induction of a Chlorophyll Radical in PSII in Photoinhibited Samples. An alternative pathway to reduce $P680^+$ involves electron donation from Cyt b_{559} mediated via a monomeric chlorophyll molecule (Thompson & Brudvig, 1988). This species, sometimes denoted Chl_Z, is an accessory chlorophyll in PSII that functions as an electron donor to $P680^+$ when other donor pathways are prevented (de Paula et al., 1985).

In Tris-washed PSII membranes, Tyr-Z is normally observed upon illumination. However, at low temperature, the electron donation from Tyr-Z to $P680^+$ is blocked. Instead, illumination of PSII at 77 K results in quantitative oxidation of Chl_Z (de Paula et al., 1985). The radical from Chl_Z^+ is normally stable for extended periods of time at 77 K (Miller & Brudvig, 1991). It can therefore be used as a quantitative probe for the functionality of P680 and the electron transfer on the donor side of PSII. We investigated the possibility to induce a stable chlorophyll radical in PSII after photoinhibition of Tris-washed samples, similar to those used for the Q_A^- and charge separation measurements in Figure 4, by illumination of the photoinhibited samples at

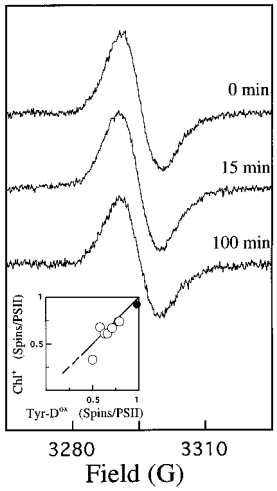

FIGURE 5: Effects of photoinhibition on the light-induced oxidation of a chlorophyll radical in Tris-washed PSII membranes. The radical EPR spectrum from Chl_Z^+ was recorded after the radical had been induced by illumination at 77 K of samples photoinhibited for various times. The spectra shown are difference spectra between the light-induced radical spectra and the spectrum in the same sample prior to illumination at 77 K (which is dominated by Tyr-D^{ox}) and represent spectra of Chl_Z^+ after photoinhibition for 0, 15, and 100 min. The inset shows the correlation between the loss of Tyr-D^{ox} and the decrease in the induction of the spectrum from Chl_Z^+ (open circles) as a consequence of photoinhibition. The filled circle represents a dark control after 100 min incubation in the dark. EPR conditions: *T* 15 K; microwave frequency 9.23 GHz; microwave power 0.5 mW; modulation amplitude 2.8 G. The size of the Chl_Z^+ signal in the control was similar to the size of the spectrum from Tyr-D^{ox} in the same sample. It therefore represents one oxidized chlorophyll per functional PSII reaction center.

77 K. Figure 5 shows the radical spectra induced by the low temperature illumination. In the non-photoinhibited control, the size of the radical corresponded to 1 radical per PSII reaction center. The EPR parameters, $g = 2.0025$ and linewidth 9.5−10 G, were typical for those of the monomeric chlorophyll (Miller & Brudvig, 1991). The size of the light-induced signal diminished with photoinhibition (Figure 5). The kinetics for the inhibition were approximately similar to those for the disappearance of Tyr-D (Figure 5, inset) and inhibition of the charge separation (Figures 4B,C). This is a logical consequence of the inhibition of the charge separation and further strengthens the conclusion that many of the photochemically active components in PSII are inactivated simultaneously (P680, pheophytin, Q_A, Tyr-D and Chl_Z).

The formation of chlorophyll radicals has also been studied during photoinhibition of NH_2OH/EDTA-washed PSII membranes that lack the Mn cluster (Blubaugh et al., 1991). During the course of photoinhibition, a light-induced radical was observed. This radical, which did not diminish faster than the amplitude of the Tyr-D^{ox}, was tentatively assigned to Chl_Z^+. However, this assignment is difficult to sustain from the published EPR spectra which do not allow precise determination of either the spectral linewidth or the *g* values (Blubaugh et al., 1991). In the same work, photoinhibition was found to give rise to a dark stable, quite wide radical with a high *g* value (linewidth ≈ 12 G, $g \approx 2.004$), which was tentatively assigned to a carotenoid cation. We have not been able to observe any similar radical in our experiments, despite efforts to create experimental conditions similar to those described by Blubaugh et al. (1991). At present, we have no explanation for this difference.

CONCLUDING REMARKS

We have presented results on the sequence of events leading to donor-side photoinhibition in Cl^--depleted and Tris-washed PSII membranes. Inhibition of the oxygen evolution in the Cl^--depleted membranes correlated with the light-induced release of 2Mn/PSII. A likely explanation for the release of Mn is the formation of a reductant during illumination, possibly H_2O_2 or O_2^- (Schröder & Åkerlund, 1986; Fine & Frasch, 1992). The disruption of the Mn cluster coincided with an increase in DPC donation and increase in the EPR signal from Tyr-Z^{ox}, reflecting a change in the environment around Tyr-Z due to the Mn release. The inactivation of Tyr-Z which seemingly is the next component to become inactivated during donor-side photoinhibition occurred on a slower time scale. This delay is interesting, and one could speculate that the Mn released could be kept reduced by H_2O_2 produced (Boussac et al., 1986) and thereby function as an electron donor to Tyr-Z for several turnovers before it becomes inactivated. This may occur in a direct reaction with H_2O_2 (Bradley et al., 1991) or O_2^- (Chen et al., 1992, 1995), leading to peroxidation or cross-linking reactions.

Alternatively, Tyr-Z^{ox}, which is very oxidizing, could oxidize components in its vicinity, leading to inhibition of the electron transfer to $P680^+$. However, the inactivation of Tyr-Z did not trigger the D1 protein for degradation. The D1 protein is instead damaged in a slower reaction that involves other light-induced species than Tyr-Z^{ox}. Seemingly, Tyr-Z is selectively inhibited. In photoinhibited samples where only 30% of Tyr-Z could be oxidized, more than 70% of the centers are still quite intact. Tyr-D^{ox}, reducible Q_A, and the charge separation reaction remain functional in those centers. Consequently, the D1 protein is correctly assembled with the D2 protein in those centers. The remaining PSII components were later photoinhibited in concert together with the degradation of the D1 protein. Thus, Q_A was not inactivated before the primary charge separation during donor-side photoinhibition. This is in contrast to the situation during acceptor-side-induced photoinhibition where the quinones sequentially become inactivated before the function of the primary charge separation is lost (Styring et al., 1990b; Vass et al., 1992a).

Although both the Mn cluster and Tyr-Z were inactivated, secondary electron transfer could occur on the donor side of PSII. This was demonstrated by the possibility to induce a donor side radical in these centers and proves that P680 is functional. The EPR features of this radical coincided with those of Chl_Z, an accessory chlorophyll in PSII (Miller & Brudvig, 1991).

It is not totally abnormal to observe Chl_Z oxidation. In case the water splitting complex or Tyr-Z are incompetent as donors to $P680^+$ (for example, at very low temperature), an alternative electron donation pathway from Cyt b_{559} to $P680^+$ via Chl_Z becomes operational. This pathway has been suggested to protect against photoinhibition at low light (Thompson & Brudvig, 1988; Barber & De Las Rivas, 1993). However, in strong light Cyt b_{559} cannot prevent accumulation of Chl_Z^+, which therefore is unable to reduce $P680^+$. The acceptor side is still operational (this holds in our experiments), which allows the quinone acceptors to efficiently drain electrons. Therefore, a strongly oxidizing equivalent may be left on P680. This might oxidize almost

anything in its environment. Potential targets for oxidation include amino acid side chains or a carotenoid that is known to be oxidized during illumination of PSII reaction centers (Klimov et al., 1990; Blubaugh et al., 1991; Telfer et al., 1991; De Las Rivas et al., 1993). We hypothesize that these secondary oxidizing reactions trigger the degradation of the D1 protein. Thus, in a PSII center with inactivated donors (Mn cluster, Tyr-Z), but with a functional acceptor side that efficiently drains electrons, long-lived $P680^+$ is the most likely species to induce oxidative damage to the D1 protein.

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