Effects of Photoinhibition on the $Q_A^-Fe^{2+}$ Complex of Photosystem II Studied by EPR and Mössbauer Spectroscopy†

Imre Vass,*,‡,§ Yiannis Sanakis,§ Cornelia Spetea,‡ and Vasili Petrouleas§

Institute of Plant Biology, Biological Research Center, Szeged, Hungary, and Institute of Materials Science, NCRS "Demokritos", Athens, Greece

Received December 2, 1994; Revised Manuscript Received January 18, 1995⊗

ABSTRACT: Effects of photoinhibition on the iron−quinone electron acceptor complex of oxygen-evolving photosystem II have been studied using low-temperature EPR and Mössbauer spectroscopy. Photoinhibition of spinach photosystem II membrane particles at 4 °C decreases the EPR signal arising from the interaction of Q_A^- with Fe^{2+} to 30% in 90 min under our conditions. The free radical EPR signal from Q_A^- induced by cyanide treatment of the iron [Sanakis, Y., et al. (1994) *Biochemistry 33*, 9922−9928] declines with the same kinetics as the $Q_A^-Fe^{2+}$ EPR signal. In contrast, Fe^{2+} is present in about 70% of the centers after 90 min of photoinhibition, as shown by its EPR-detected interaction with NO and by its Mössbauer absorption. Complete oxidation of this Fe^{2+} population to Fe^{3+} by ferricyanide is possible only in the presence of glycolate, which lowers the redox potential of the Fe^{3+}/Fe^{2+} couple. In a fraction of PSII centers, which reach 30% after 90 min of photoinhibition, the iron cannot be detected. It is concluded that photoinhibition of oxygen-evolving photosystem II affects both Q_A and Fe^{2+}. However, the photoinhibitory impairment of the Q_A redox functioning precedes the modification of the non-heme iron. In a considerable portion of the photoinhibited centers, which do not have functional Q_A, the non-heme iron is still present and redox active, but its redox potential is increased relative to that in the normal centers. This is probably due to a minor modification of the bicarbonate ligation site. In the rest of the photoinhibited centers, the loss of Q_A function is accompanied by a severe modification or release of the non-heme iron.

Beyond being the ultimate driving force of photosynthesis and its important regulatory factor, light is also a major source of damage to oxygenic photosynthetic organisms. The main target for light-induced damage is photosystem II (PSII),[1] leading to impairment of electron transport and degradation of reaction center subunits, in particular the so-called D1 protein [reviewed recently by Andersson and Styring (1991); Barber & Andersson, 1992; Aro et al., 1993].

The multifunctional pigment−protein complex of PSII is embedded in the thylakoid membrane and catalyzes the light-driven oxidation of water and the reduction of the plastoquinone pool [reviewed recently by Andersson and Styring (1991); Debus, 1992]. The redox components required for water oxidation and plastoquinone reduction are ligated by the heterodimer of the D1 and D2 proteins, which form the reaction center of PSII. The light energy captured by the antenna pigments is transferred to the primary electron donor P_{680} and induces the transfer of an electron from P_{680} to the primary electron acceptor, pheophytin (Pheo). This primary charge separation is stabilized by fast electron transfer from $Pheo^-$ to the first (Q_A) and second (Q_B) quinone electron acceptors and from the water-oxidizing complex to P_{680}^+ via the redox-active tyrosine residue (Tyr-Z). The reaction center complex also contains a redox-active non-heme iron, which is located between the Q_A and Q_B acceptors.

It is widely accepted that different molecular mechanisms of photoinhibition exist, depending on experimental conditions and biological material. Two types of photoinhibition have been well defined *in vitro*: In PSII membranes in which electron donation from water is inhibited (by Tris washing, NH_2OH treatment, or Cl^- depletion), donor side-mediated photoinhibition is observed (Theg et al., 1986; Callahan et al., 1986; Jegerschöld et al., 1990; Blubaugh et al., 1991; Eckert et al., 1991). In this case, the electron transfer from Tyr-Z to P_{680}^+ is impaired (Blubaugh et al., 1991), but the primary charge separation remains functional for a much longer time (Blubaugh et al., 1991; Eckert et al., 1991). In PSII centers in which electron donation from water is initially intact, photoinhibition is initiated at the acceptor side, at the level of the quinone acceptors (Setlik et al., 1990; Styring et al., 1990; Kirilovsky & Etienne, 1991; Vass et al. 1992; Vass & Styring, 1993). The occurrence of the acceptor side mechanism is well documented under anaerobic or very reducing conditions and includes the full reduction of the plastoquinone pool and Q_B, followed by the generation of highly stable, singly and doubly reduced Q_A species (Q_AH and Q_AH_2, respectively) (Setlik et al., 1990; Styring et al., 1990; Vass et al., 1992; Vass & Styring, 1993) and finally the release of Q_AH_2 from its binding site (Styring et al., 1990;

† This work was supported by research grants from the Hungarian Academy of Sciences (OTKA 1/III-888). I.V. was supported by a PECO'93 travel grant to Greece. V.P. acknowledges EC support through the HCM network "MASIMO".

* Corresponding author: Institute of Plant Biology, Biological Research Center, P.O. Box 521, H-6701 Szeged, Hungary. Fax: 36-62-433-434.

‡ Biological Research Center.

§ NCRS "Demokritos".

⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1995.

[1] Abbreviations: Chl, chlorophyll; cyt *b*-559, cytochrome *b*-559; EPR, electron paramagnetic resonance; PQ, plastoquinone; PS II, photosystem II; Pheo, pheophytin; Q_A and Q_B, the first and second quinone electron acceptors of PSII; P_{680}, the reaction center chlorophyll of PSII; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tyr-Z and Tyr-D, redox-active tyrosine residues of PSII.

0006-2960/95/0434-4434$09.00/0

Vass et al. 1992; van Mieghem et al., 1989; Koivuniemi et al., 1993). The primary charge separation remains functional after the acceptor side modifications (Styring et al., 1990), and illumination of the PSII centers promotes the formation of triplet P_{680} via recombination of $P_{680}^+Pheo^-$ (Vass et al., 1992, 1993; Vass & Styring, 1993; Kirilovsky et al., 1994).

Photoinhibition of thylakoids and PSII membranes under aerobic conditions leads to the loss of the EPR signal from $Q_A^-Fe^{2+}$ (Styring et al., 1990; Gleiter et al., 1992; van Wijk et al., 1992; Kirilovsky et al., 1994). This effect has been interpreted as the impairment of Q_A function by double reduction, and the idea has been put forward that the acceptor side mechanism dominates the photoinhibition of oxygen-evolving thylakoids and PSII membranes in aerobic conditions (Vass et al., 1992; van Wijk et al., 1992). Triplet P_{680}, which is expected to be formed upon illumination of the acceptor side-modified centers, facilitates the production of extremely reactive singlet oxygen and could be involved in the damage of the D1 protein. This idea is fully supported by the direct detection of singlet oxygen in aerobically photoinhibited oxygen-evolving material (Hideg et al., 1994a,b) and by the protection of singlet oxygen scavengers against photoinduced damage (Mishra et al., 1994). However, the yield of triplet P_{680} formation is low in the aerobically photoinhibited samples (Vass et al., 1992; van Wijk et al., 1992; Kirilovsky et al., 1994), despite the apparent ability of the centers to produce primary charge separation (Allakhverdiev et al., 1987; Styring et al., 1990; van Wijk et al., 1992). In addition, triplet P_{680} formation is suppressed if anaerobic photoinhibition is performed in the presence of artificial acceptors (Kirilovsky et al., 1994). These findings were interpreted as indicating that oxygen, acting as an acceptor, may prevent the overreduction of Q_A, and thus aerobic photoinhibition is not dominated by the acceptor side mechanism (Kirilovsky et al., 1994). The observed loss of the $Q_A^-Fe^{2+}$ EPR signal was suggested to be related to changes in the properties of Fe^{2+} triggered by events occurring at the donor side in the reaction center, instead of the direct impairment of the Q_A function (Kirilovsky et al., 1994).

The properties of Fe^{2+} are indeed susceptible to modifications by strong light, and the ability of ferricyanide to oxidize it to Fe^{3+} is decreased by aerobic photoinhibition (Haag et al., 1992; Gleiter et al., 1992). However, the kinetic and functional relationship between the loss of Q_A function and the modification of the non-heme iron has not been studied in detail.

The goal of this work is to understand the relationship between the effects of photoinhibition on Q_A and Fe^{2+} under aerobic conditions in oxygen-evolving material. For this purpose, we applied low-temperature EPR measurements in combination with Mössbauer spectroscopy and followed the changes induced by photoinhibition in the properties of the two species separately. The results demonstrate that impairment of Q_A redox functioning precedes the modification of the non-heme iron.

MATERIALS AND METHODS

PSII membranes for EPR measurements were isolated from spinach as described previously (Vass & Strying, 1992) and were stored at −80 °C until use in 0.4 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl, and 40 mM Hepes (pH 7.5). PSII membranes for Mössbauer measurements, isolated from spinach plants grown hydroponically in a medium enriched in ^{57}Fe to approximately 90%, were kindly provided by Dr. Bruce Diner (Wilmington, DE).

Samples of 4.5 mg of Chl/mL were photoinhibited in EPR tubes at 4−6 °C using heat-filtered white light of approximately 5000 μE m^{-2} s^{-1}. In order to provide as homogeneous an illumination of the samples as possible, the EPR tubes were turned by 90° every 2 min. After photoinhibition, the samples were dark adapted for 10 min at room temperature and subjected to further treatments in order to monitor the extent of damage to Q_A and Fe^{2+} independently. For measurement of the $Q_A^-Fe^{2+}$ EPR signal, 100 mM sodium formate was added and allowed to react for 40 min in the dark. To produce the free radical EPR signal of Q_A^-, samples were dark incubated with 325 mM sodium cyanide at pH 8.0 for 80 min at 4 °C and with 50 mM sodium dithionite for an additional 10 min. NO binding to the iron was induced by slowly bubbling the samples with a gaseous mixture of NO and N_2 (in a 2:1 ratio), followed by a 45 min anaerobic incubation in the dark at 4 °C. Oxidation of Fe^{2+} was achieved by incubation with 5 mM ferricyanide at pH 7.5 for 30 min in the dark at room temperature. Glycolate treatment was performed by incubating the ferricyanide-treated samples with 40 mM glycolate for an additional 2 h at 4 °C.

X-band low-temperature EPR spectra were recorded at 9.42 GHz microwave frequency with a Bruker ER 200D spectrometer interfaced to a PC and equipped with an Oxford ESR9 cryostat and temperature controller, an NMR gaussmeter, and an Anritsu MF76A microwave frequency counter. Illumination of the EPR samples was performed in an unsilvered dewar, with a projector lamp through a heat-absorbing $CuSO_4$ filter at 77 (in liquid nitrogen) or 200 K (in acetone cooled by liquid nitrogen).

Mössbauer measurements were obtained with a conventional constant acceleration spectrometer and $^{57}Co(Rh)$ source at 77 K. The sample contained approximately 12 mg of Chl in total.

Light-induced changes in the protein structure of PSII were followed by SDS−PAGE on a 12−17% linear acrylamide gradient gel containing 6 M urea, as described previously (Friso et al., 1993). For detection of the D1 reaction center protein by immunoblotting, the resolved proteins were transferred to a nitrocellulose filter (Sartorius 45 μm) by electroblotting and identified by using a polyclonal antibody raised against the D1 protein (a kind gift of Dr. Roberto Barbato, Dept. of Biology, University of Padova). The reaction was visualized by a colorimetric method as described previously (Friso et al., 1993), and the immunodecorated blots were analyzed by a Bio-Rad 1650 densitometer attached to a Hewlett-Packard integrator (HP 33941).

RESULTS

The $Q_A^-Fe^{2+}$ EPR Signal. Photoinhibition of oxygen-evolving PSII membranes results in the rapid loss of the $Q_A^-Fe^{2+}$ EPR signal formed after photoinhibition by chemical reduction or by illumination at 200 K. The signal can be most easily monitored by treatment of the samples with formate (Vermaas & Rutherford, 1984). Under our experimental conditions, the signal decreases to about one-half of its original size after 30 min of photoinnibition and to less

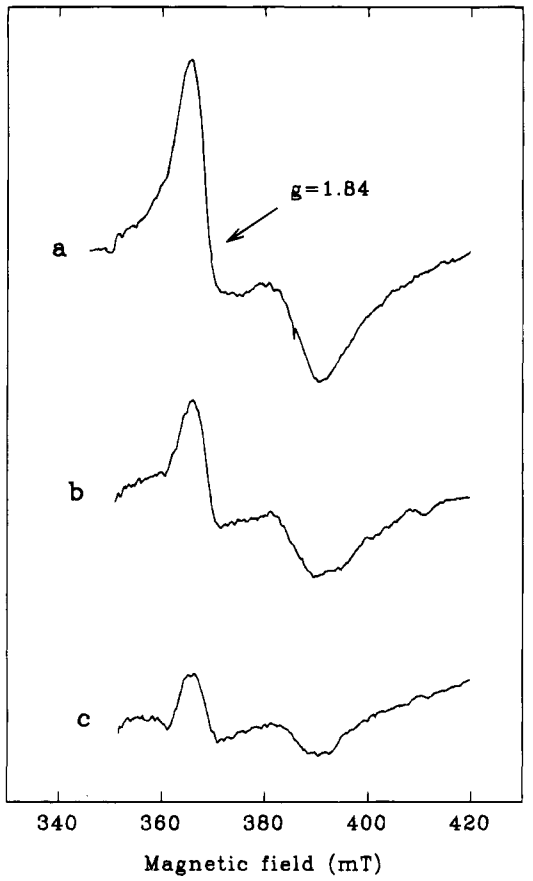

FIGURE 1: Effect of photoinhibition on the $Q_A^-Fe^{2+}$ EPR signal of PSII membrane particles. Samples were photoinhibited for 0 (a), 30 (b), and 90 min (c) at 4 °C. Reduction of Q_A was induced in the photoinhibited samples by 10 min of illumination at −80 °C in the presence of 100 mM sodium formate, which was added after the photoinhibitory treatment. The curves shown represent light minus dark difference spectra. EPR conditions: temperature = 4.2 K; microwave power = 32 mW; modulation amplitude = 3.2 mT.

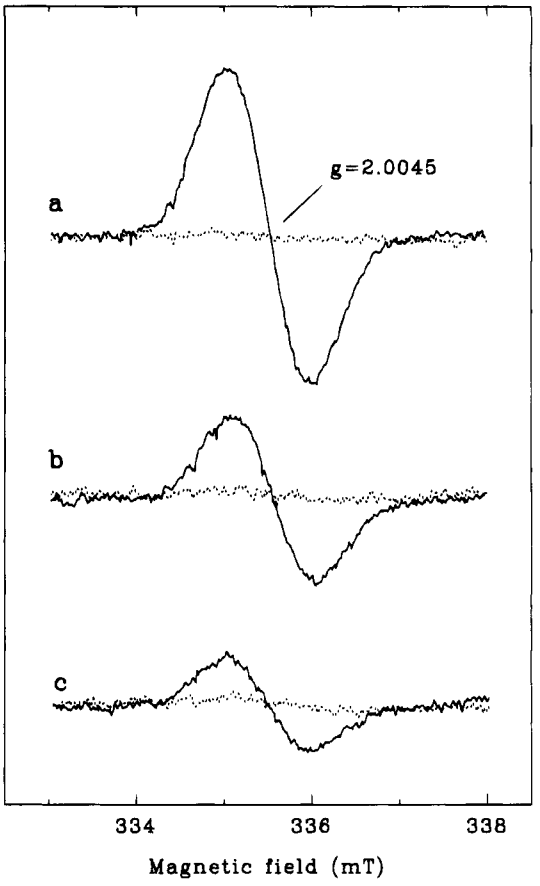

FIGURE 2: Effect of photoinhibition on the decoupled Q_A^- EPR signal. Samples were photoinhibited for 0 (a), 30 (b), and 90 min (c). Conversion of Fe^{2+} to the magnetically non-interacting low-spin ($S = 0$) state was achieved by treating the photoinhibited samples with 325 mM sodium cyanide. This was followed by the addition of 50 mM sodium dithionite, in order to induce dark reduction of Q_A and to abolish Signal II_{slow} in the $g = 2$ region by reducing Tyr-D^+. The solid curves measured after this treatment represent the EPR signal of decoupled Q_A^-. The effect of dithionite treatment alone is shown by the dotted curves. EPR conditions: temperature = 15 K; microwave power = 200 nW; modulation amplitude = 0.4 mT.

than 30% after 90 min (Figure 1). In agreement with previous results (Styring et al., 1990), the inhibition of the $Q_A^-Fe^{2+}$ signal occurs in parallel with the inhibition of the multiline EPR signal from the S_2 state of the water-oxidizing complex (not shown), indicating that the loss of oxygen evolution is correlated with the impaired redox functioning of the quinone−iron acceptor complex.

Diminution of the EPR signal from $Q_A^-Fe^{2+}$ during aerobic photoinhibition is expected to reflect the impairment of the Q_A function (Styring et al., 1990; Vass et al., 1992; van Wijk et al., 1992), but in principle it can be induced by the loss or modification of Fe^{2+}. This uncertainty can only be clarified by measuring the presence and redox activity of Q_A and the iron independently.

The Free Radical-Type Q_A^- EPR Signal. It has recently been demonstrated that treatment of PSII membranes with cyanide converts the high-spin ($S = 2$) state of the Fe^{2+} into the low-spin ($S = 0$) state and, therefore, eliminates its magnetic interaction with Q_A^- (Sanakis et al., 1994). Under these conditions, a free radical-type EPR signal arises from Q_A^- at $g = 2.0045$, which makes it possible to monitor Q_A reduction independently of its interaction with Fe^{2+}. The effect of photoinhibition on the decoupled Q_A^- signal is examined in Figure 2. The Q_A^- signal decays in parallel with the $Q_A^-Fe^{2+}$ signal (Figure 1), decreasing to about 30% after 90 min of photoinhibition. It is also important to note that, prior to the cyanide treatment, there is no EPR signal observed from decoupled Q_A^- in the photoinhibited samples (Figure 2). This indicates that photoinhibition itself does not induce the breakup of the magnetic interaction between Q_A^- and Fe^{2+}.

The NO−Fe^{2+} EPR Signal. The presence of Fe^{2+} in the PSII reaction center can be monitored independently of its interaction with Q_A^- by measuring the ability of NO to bind Fe^{2+} (Petrouleas & Diner, 1990). The NO−Fe^{2+} complex gives rise to an EPR signal in the $g = 4$ region (Figure 3). In the photoinhibited samples, the NO−Fe^{2+} signal gradually decreases, but after 90 min of photoinhibition its size is still about 70% of that in the control. By comparing Figure 3 with Figure 1 (or Figure 2) it is evident that the amount of Fe^{2+} is decreased to a substantially smaller degree than the amount of reducible Q_A. It is also instructive to examine the light sensitivity of the NO−Fe^{2+} signal. Upon illumination of the non-photoinhibited control, Q_A is reduced to Q_A^-. The magnetic coupling between Q_A^- and the NO−Fe^{2+} complex leads to the almost complete disappearance of the NO−Fe^{2+} EPR signal (Petrouleas & Diner, 1990). In the photoinhibited samples, the light-sensitive population of the NO−Fe^{2+} signal decreases (Figure 3). It is notable that the

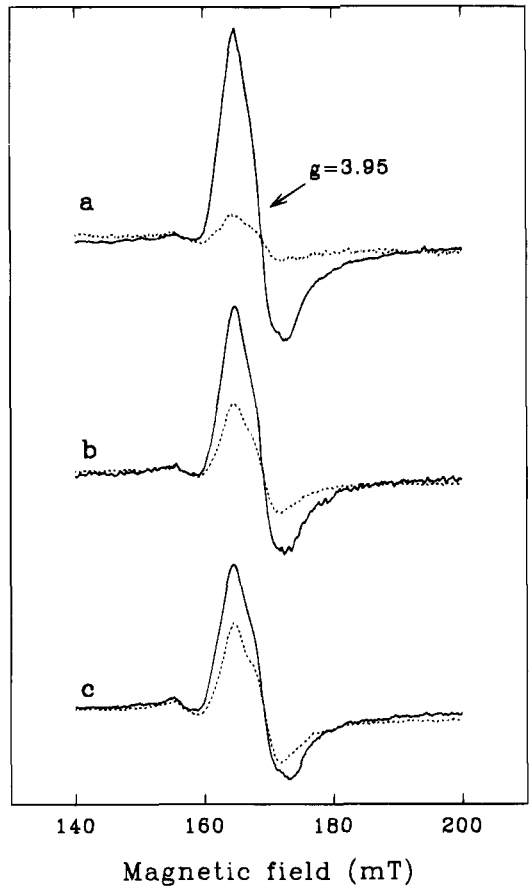

FIGURE 3: Effect of photoinhibiton on the NO−Fe^{2+} EPR signal. The samples were photoinhibited for 0 (a), 30 (b), and 90 min (c) and then treated with NO for 45 min at 4 °C in the dark. The NO-treated samples were subsequently illuminated for 4 min at −80 °C to produce the EPR-silent $Q_A^-Fe^{2+}$−NO state. The solid and dashed lines show the NO−Fe^{2+} EPR signal before and after illumination, respectively. EPR conditions: temperature = 5 K; microwave power = 32 mW; modulation amplitude = 1.6 mT.

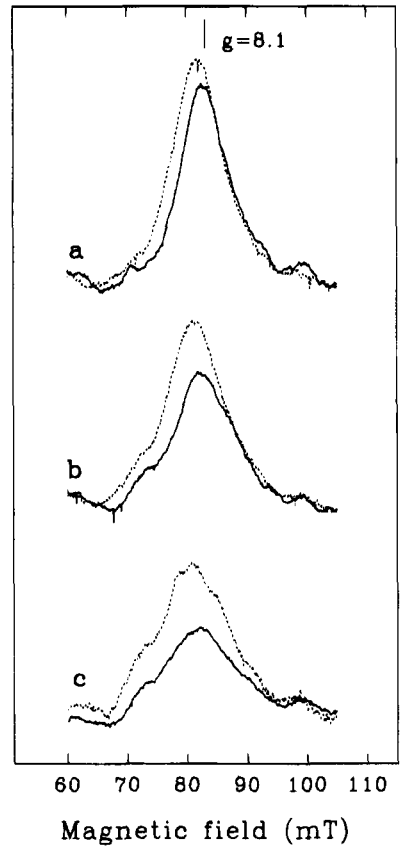

FIGURE 4: Effect of photoinhibition on the Fe^{3+} EPR signal in the g = 8 region of PSII membrane particles. The samples were photoinhibited for 0 (a), 30 (b), and 90 min (c). Oxidation of the non-heme iron from the Fe^{2+} to the Fe^{3+} form was induced by incubating the samples in the presence of 5 mM potassium ferricyanide at pH 7.5 for 30 min in the dark at room temperature. The solid curves shown are the spectra obtained after the incubation with ferricyanide. The samples were subsequently incubated with 40 mM glycolate for 2 h at 4 °C, and the EPR spectra were recorded again (dotted lines). The curves shown represent oxidized minus initially untreated (reduced) difference spectra. EPR conditions: temperature = 4.2 K; microwave power = 8 mW; modulation amplitude = 1.6 mT.

light-insensitive fraction of the signal does not change shape, indicating that in this population of centers the symmetry of the NO−Fe^{2+} environment [nearly axial (Petrouleas & Diner, 1990)] is not modified. It is likely, therefore, that the loss of magnetic interaction with Q_A^- is due to photoinhibitory damage to the Q_A site.

The Fe^{3+} EPR Signal. The presence and redox activity of the non-heme iron can also be examined by measuring the EPR signal that arises from its oxidized form (Fe^{3+}). Treatment of PSII membranes with 5 mM ferricyanide at pH 7.5 is expected to convert all of the Fe^{2+} to Fe^{3+}. This gives rise to characteristic EPR signals with a prominent contribution at around g = 8 (Diner & Petrouleas, 1987). This signal decreases in the photoinhibited samples, reaching about 50% after 90 min (Figure 4), while no alternative Fe^{3+} signals can be detected in an expanded spectral region (not shown). The extent to which Fe^{3+} formation is decreased exceeds the decrease in Fe^{2+} as detected by NO binding (Figure 3). After the samples are treated with glycolate, which has been demonstrated to bind to the iron and lower the redox potential of the Fe^{3+}/Fe^{2+} couple (Deligiannakis et al., 1994), the Fe^{3+} signal is substantially enhanced in the photoinhibited samples (Figure 4). In contrast, in the nontreated control, besides the expected small shift in the signal position (Deligiannakis et al., 1994) only a slight increase in the Fe^{3+} EPR signal is induced by the glycolate treatment (Figure 4). These observations indicate that in some of the centers photoinhibition induces a redox potential increase in the non-heme iron or, less likely, that the Fe^{3+} signals are modified beyond recognition. It is also clear that in a fraction of the photoinhibited centers, about 30% after 90 min, the g = 8 Fe^{3+} signal is not formed even in the presence of glycolate, indicating severe modifications in the environment around the iron.

The preceding results indicate that after 90 min of photoinhibition about 70% of the centers retain the ability to bind exogenous ligands (NO, glycolate) to the iron and show unmodified EPR signals. Most likely, therefore, the rapid decay of the $Q_A^-Fe^{2+}$ and decoupled Q_A^- signals is not due to the loss of the ability of the iron to bind formate and cyanide, respectively. It is instead due, as discussed in the following, to a rapid impairment of the Q_A redox activity.

The Mössbauer Signal of Fe^{2+}. Changes in the state of the non-heme iron during photoinhibition were also followed by Mössbauer spectroscopy. In the non-photoinhibited control, contributions from cyt *b*-559 and from the non-heme iron can be distinguished (Figure 5) (Petrouleas & Diner, 1982; Petrouleas et al., 1992). There are two Mössbauer components of high-spin Fe^{2+} (component 1 and component

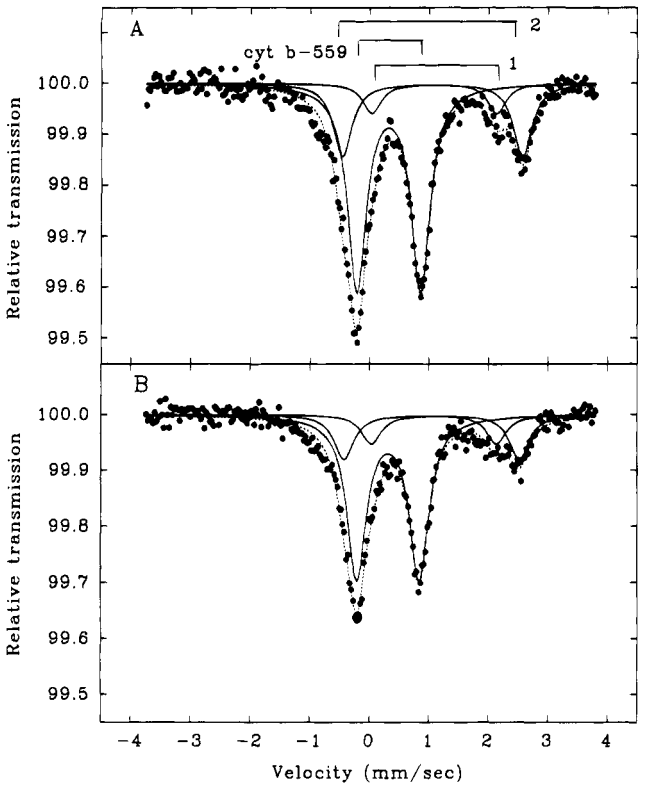

FIGURE 5: Effect of photoinhibition on the Mössbauer spectrum of PSII membrane particles. The Mössbauer spectrum was first recorded in the untreated sample at 77 K (A), which was then subjected to photoinhibition for 90 min under the same conditions applied for the EPR samples, followed by a new Mössbauer measurement (B). The experimental data are represented by dots, whereas the solid and dashed lines show the deconvoluted quadruple pair components and the fitted spectra, respectively.

2), which represent two populations of the non-heme iron in a somewhat different environment (Petrouleas et al., 1992). In the photoinhibited samples the same components are present, but their intensity is smaller than in the control. Although the relative normalization of the data is approximate due to the loss of sample material (less than 10%) after the photoinhibitory treatment, some interesting observations can be made. (i) The loss of the non-heme iron (Fe^{2+}) absorption is relatively small, 20−30%, in agreement with the EPR results. (ii) The relative contribution of component 1 to the non-heme iron absorption increases following photoinhibition, in accord with the Fe^{3+} EPR experiments and with previous data (Petrouleas et al., 1992), which indicate that component 1 is not oxidizable by ferricyanide. (iii) The decrease in the cyt *b*-559 signal probably indicates the oxidation of part of the high-potential cyt *b*-559, which is in agreement with previous EPR data (Styring et al., 1990).

D1 Protein Degradation. D1 protein degradation is a well documented consequence of photoinhibition [see Andersson and Styring (1991)] and is believed to occur in two consecutive steps in thylakoids and PSII membranes (Aro et al., 1990; Richter et al., 1990). In the first step, the protein is damaged in a light-dependent process that triggers it for degradation. The actual degradation does not require light and is retarded at low temperature (Aro et al., 1990; Richter et al., 1990). In accord with this, the D1 protein is barely lost under the conditions of our photoinhibitory treatment (4−6 °C) (Figure 6), even though in some of the photoinhibited centers it is most likely damaged.

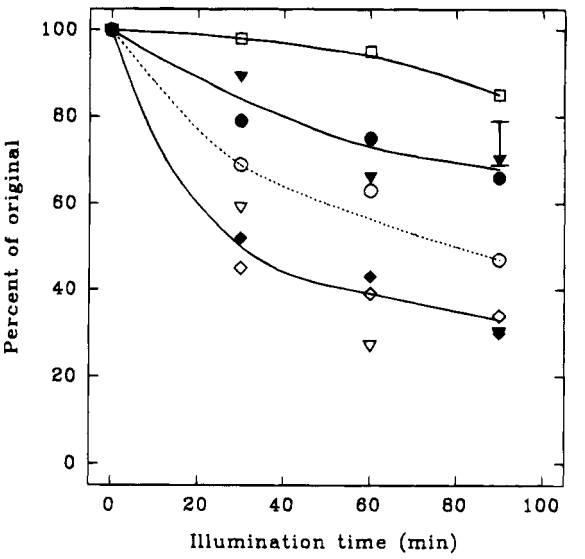

FIGURE 6: Time course of PSII inactivation during photoinhibition. The amplitude of the EPR signals, obtained as for Figures 1−4, is plotted as a function of photoinhibition time: $Q_A^-Fe^{2+}$ obtained after −80 °C illumination (◆); decoupled Q_A^- obtained after treatment with 325 mM cyanide (◇); $NO-Fe^{2+}$ in the dark (▼); light-sensitive $NO-Fe^{2+}$ obtained from the dark minus light difference spectra (▽); Fe^{3+} after treatment with 5 mM ferricyanide (○); Fe^{3+} in the presence of ferricyanide and 40 mM glycolate (●); amount of the D1 reaction center protein (□). The vertical bar indicates the limits of Fe^{2+} loss after 90 min of photoinhibition as estimated from the Mössbauer measurements.

Kinetics of Photoinactivation. The decay course of the various signals during photoinhibition is summarized in Figure 6. The free radical-type Q_A^- signal, which is observed after Fe^{2+} is converted into the magnetically non-interacting low-spin ($S = 0$) state by cyanide treatment (Sanakis et al., 1994), decays in parallel with the $Q_A^-Fe^{2+}$ signal (Figure 6). This decay course is very similar to the loss of the light-sensitive fraction of the $NO-Fe^{2+}$ signal (Figure 6). In contrast, the full size of the $NO-Fe^{2+}$ signal, which is the measure of the total amount of Fe^{2+} that can bind NO, is decreased significantly more slowly than the $Q_A^-Fe^{2+}$ signal (Figure 6). The maximal size of the Fe^{3+} EPR signal, obtained in the presence of ferricyanide plus glycolate (Figure 6), follows the same decay course as the NO-detectable Fe^{2+}. The fraction of the Fe^{3+} signal that is induced by ferricyanide alone (Figure 6) is lost faster than the full Fe^{3+} or $NO-Fe^{2+}$ signals, but slower than the $Q_A^-Fe^{2+}$ and free radical Q_A^- signals. The results also show that there is an increasing fraction of photoinhibited centers (reaching about 30% after 90 min) in which Fe^{2+} or Fe^{3+} cannot be detected by EPR.

The decrease in Mössbauer absorption intensity in the non-heme iron range is in accord with the loss of EPR-detectable iron after prolonged photoinhibition. The centers that lose components 1 and 2 in the Mössbauer spectrum are likely to correspond to those in which the environment of the iron is drastically altered and the $NO-Fe^{2+}$ and Fe^{3+} EPR signals cannot be detected. Considering the ca. 30% upper limit for the decrease in Mössbauer intensity, there is reasonable qualitative agreement with the EPR results (Figure 6).

The total amount of the D1 protein decays very slowly (Figure 6), indicating that the cause of the loss of the EPR and Mössbauer signals is not the destruction of the reaction center complex.

DISCUSSION

Photoinhibition Damages Q_A Faster Than Fe^{2+}. As has been observed previously and confirmed by us here, the loss of the $Q_A^-Fe^{2+}$ EPR signal is the primary event in aerobic photoinhibition. Due to its origin as a magnetic interaction, the observed diminution of the $Q_A^-Fe^{2+}$ signal can, in principle, be initiated either by the impairment of Q_A^- formation or by the modification or release of Fe^{2+}. Among these alternatives, the impairment of Q_A function by double reduction and subsequent release from its binding site was originally suggested (van Mieghem et al., 1989; Styring et al., 1990; Vass et al., 1992). However, the susceptibility of Fe^{2+} to modifications by strong light has also been shown (Haag et al., 1992; Gleiter et al., 1992), and the possibility that this modification is responsible for the loss of the $Q_A^-Fe^{2+}$ signal has been raised (Kirilovsky et al., 1994). In order to obtain independent information about the two components of the quinone–iron acceptor complex in photoinhibited PSII, we probed Q_A and Fe^{2+} separately from each other.

The decay of the $Q_A^-Fe^{2+}$ EPR signal during photoinhibition is not accompanied by the appearance of modified EPR signals. In particular, no free radical Q_A^- signal is induced in the photoinhibited samples, similar to what has been observed after removal of the iron by chemical treatments (Klimov et al., 1980). When, however, the iron is converted to the magnetically inactive low-spin state by the recently reported cyanide treatment (Sanakis et al., 1994), the free radical Q_A^- signal is found to decay in parallel with the $Q_A^-Fe^{2+}$ signal. These observations suggest that the iron is not lost prior to the inactivation of Q_A.

The number of centers that retain functional Fe^{2+} after photoinhibition was monitored by following the binding of NO to Fe^{2+} and by the ability to oxidize Fe^{2+} to Fe^{3+}. The total amplitude of the NO–Fe^{2+} and Fe^{3+} EPR signals is decreased significantly more slowly than the $Q_A^-Fe^{2+}$ signal. Thus, evidently there are photoinhibited PSII centers that retain redox-active Fe^{2+}, but do not contain normally functioning Q_A. The Mössbauer absorption intensity in the non-heme iron range is in accord with the EPR data and shows that most of the high-spin Fe^{2+} is present after prolonged photoinhibition.

The preferential inactivation of Q_A is further corroborated by the observation that the light sensitivity of the NO–Fe^{2+} signal, which is due to the magnetic interaction of the NO–Fe^{2+} complex with Q_A^- (Petrouleas & Diner, 1990), decreases with photoinhibition. The reason is that photoinhibition impairs Q_A reduction in an increasing population of centers in which the NO–Fe^{2+} complex is intact. Another observation, which probably relates with faster damage of Q_A, is that, in the photoinhibited samples, light-induced reduction of Fe^{3+} requires a longer period of illumination than in the nontreated control (not shown). Normally, the reduction of iron is fast and proceeds via Q_A (Diner & Petrouleas, 1987).

Taken together, these results show that it is the impaired function of Q_A that initiates the loss of the $Q_A^-Fe^{2+}$ signal during aerobic photoinhibition. This is in agreement with the previously suggested double reduction of Q_A (van Mieghem et al., 1989; Styring et al., 1990; Vass et al., 1992) and does not support the idea of a preferential loss of Fe^{2+}.

Photoinhibitory Effects on the Iron. The decreased oxidizability of Fe^{2+}, as detected by measuring the ferricyanide-induced Fe^{3+} EPR signal in the $g = 8$ region, has already been reported by Gleiter et al. (1992). The effect has been suggested to reflect decreased accessibility of the oxidant to the iron site or increased redox potential of the iron. Here we demonstrate that, in the photoinhibited samples, Fe^{2+} oxidation is reversed in part by glycolate, which lowers the redox potential of the iron (Deligianniakis et al., 1994).

These results can be easily reconciled with the NO experiments, if we assume that in a population of the photoinhibited centers the iron site is modified slightly to induce a higher midpoint potential. NO or glycolate addition restores uniform behavior to a large fraction of centers (70% after 90 min of photoinhibition), identical to that of the control. It is notable that the NO–Fe^{2+} or Fe^{3+} (glycolate) signal shape, which is very sensitive to the ligand field symmetry, is identical to that of the non-photoinhibited control samples. Both NO and glycolate bind to the iron in competition with bicarbonate (Diner & Petrouleas, 1990; Petrouleas et al., 1994). Thus, it is possible that the increase in the midpoint potential of the iron is due to a modification in bicarbonate binding similar to what has been implied for the increase in the midpoint potential of the nontreated iron below pH 6.3 (Diner & Petrouleas, 1987; Petrouleas et al., 1994).

The increased midpoint potential in a fraction of the non-heme iron is also consistent with the Mössbauer data. The two components of the non-heme iron are assigned to two different conformations of the Fe^{2+}. These populations seem to have different redox potentials: the E_m of component 1 is more positive than that of component 2 (Petrouleas et al., 1992). The relative increase in component 1 compared to component 2 induced by photoinhibition is in agreement with an increase in the redox potential of the iron in some of the centers and may arise from the same population in which ferricyanide can oxidize Fe^{2+} only after glycolate treatment.

The results also show that there is an increasing fraction of photoinhibited centers (about 30% after 90 min) in which Fe^{3+} is not induced, even by ferricyanide plus glycolate. In approximately the same number of centers, Fe^{2+} cannot be detected by NO binding or by Mössbauer spectroscopy. Thus, it is likely that in the later phase of photoinhibition the modification of the non-heme iron is more serious than a redox potential increase. Such a drastic change could be induced by a structural modification of the PSII centers, which leads to the alteration of the protein environment around the iron or to its release from the binding site.

It is of note that some inhomogeneity in the extent of photoinhibition cannot be avoided due to the photoinhibition of high concentration samples in EPR tubes. Such inhomogeneity can affect the rate of photoinhibition, i.e., PSII units in the inner part of the sample will be inhibited later than centers in the outer part of the sample. However, it is unlikely that the differential response of the two components of the $Q_A^-Fe^{2+}$ complex, observed after a given time of photoinhibition, is influenced by the partly inhomogeneous illumination. Thus, sample inhomogeneity is not expected to change the preceding interpretation of the results.

Implications for D1 Protein Damage. The binding niche of the non-heme iron is formed by four histidine residues of the D1 and D2 proteins [D1His215, D1His272, D2His214,

and D2His268 (Trebst, 1986; Michel & Deisenhofer, 1986)]. Under the conditions of our photoinhibitory treatment (4−6 °C), the D1 protein is barely degraded, even though in some of the photoinhibited centers it is most likely damaged and triggered for degradation. The nature of the triggering step is not clarified, but in aerobic photoinhibition it is likely to be induced by singlet oxygen (Vass et al., 1992; Mac Pherson et al., 1993; Hideg et al., 1994a,b), which can attack amino acid residues and pigment components of the PSII reaction center. This oxidative attack may induce a conformational change in D1, exposing its cleavage site to the protease. The cleavage region of D1 is located in the acceptor side loop between the fourth and fifth helices in oxygen-evolving material (Arntz & Trebst, 1986). This site is relatively close to the iron binding ligands of D1 near the lumenal ends of the fourth and fifth helices. Thus, a conformational change related to the triggering process may easily alter the protein environment around the iron. In that case, the population of centers in which the iron cannot be detected would represent centers with the D1 protein triggered for degradation.

Light-induced degradation of the D2 protein is much less pronounced than that of D1 [see Andersson and Styring (1991)]. However, a structural change in the D2 protein may also occur as a consequence of photoinhibition. Q_A is believed to be bound by the D2 protein, and one of the Fe^{2+}-ligating histidines (D2His214) is suggested to form an H-bond with Q_A (Diner et al., 1991). In acceptor side photoinhibition, Q_A is doubly reduced and subsequently released from the binding site (Styring et al., 1990; Vass et al., 1992; Koivuniemi et al., 1993). This event, which does not occur in normally functioning PSII, has been suggested to induce a structural change (Vass et al., 1992) and may easily affect the nearby iron binding region. In that case, the population of centers that lack detectable iron would represent centers from which Q_A is released. Our data and the available literature results at present do not permit a clear distinction between the two possibilities.

Conclusions. The results of this study show that both components of the quinone−iron electron acceptor complex of PSII are damaged in aerobic photoinhibition. However, the redox function of Q_A is impaired faster than the modification of the non-heme iron. From this finding, it follows that the inactivation of Q_A cannot be due to a preferential effect on the iron, but most likely is caused by double reduction as proposed previously. The occurrence of this specific acceptor side modification provides support for an important contribution of the acceptor side mechanism in the aerobic photoinhibition of oxygen-evolving PSII membranes.

ACKNOWLEDGMENT

We thank Dr. Roberto Barbato (Dept. of Biology, University of Padova) for his generous gift of the D1 antibody.

REFERENCES

Allakhverdiev, S. I., Setlikova, E., Klimov, V. V., & Setlik, I. (1987) *FEBS Lett. 226*, 186−190.
Andersson, B., & Styring, S. (1991) *Curr. Top. Bioenerg. 16*, 1−81.
Arntz, B., & Trebst, A. (1986) *FEBS Lett. 194*, 43−49.
Aro, E.-M., Hundal, T., Carlberg, I., & Andersson, B. (1990) *Biochim. Biophys. Acta 1019*, 269−275.
Aro, E.-M., Virgin, I., & Andersson, B. (1993) *Biochim. Biophys. Acta 1143*, 113−134.
Barber, J., & Andersson, B. (1992) *Trends Biochem. Sci. 17*, 61−66.
Blubaugh, D. J., Atamian, M., Babcock, G. T., Golbeck, J. H., & Cheniae, G. M. (1991) *Biochemistry 30*, 7586−7597.
Callahan, F. E., Becker, D. W., & Cheniae, G. M. (1986) *Plant Physiol. 82*, 261−269.
Debus, J. R. (1992) *Biochim. Biophys. Acta 1102*, 269−352.
Deligiannakis, Y., Petrouleas, V., & Diner, B. A. (1994) *Biochim. Biophys. Acta 1188, 260−270.*
Diner, B. A., & Petrouleas, V. (1987) *Biochim. Biophys. Acta 895*, 107−125.
Diner, B. A., & Petrouleas, V. (1990) *Biochim. Biophys. Acta 1015*, 141−149.
Diner, B. A., Petrouleas, V., & Wendoloski, J. J. (1991) *Physiol. Plant 81*, 423−436.
Eckert, H.-J., Geiken, B., Bernarding, J., Napiwotzki, A., Eichler, H.-J., & Renger, G. (1991) *Photosynth. Res. 27*, 97−108.
Friso, G., Spetea, C., Giacometti, G. M., Vass, I., & Barbato, R. (1993) *Biochim. Biophys. Acta 1144*, 265−270.
Gleiter, H. M., Nugent, J. H. A., Haag, E., & Renger, G. (1992) *FEBS Lett. 313*, 75−79.
Haag, E., Gleiter, H. M., & Renger, G. (1992) *Photosynth. Res. 31*, 113−126.
Hideg, É., Spetea, C., & Vass, I. (1994a) *Photosynth. Res. 39*, 191−199.
Hideg, É., Spetea, C., & Vass, I. (1994b) *Biochim. Biophys. Acta 1186*, 143−152.
Jegerschöld, C., Virgin, I., & Styring, S. (1990) *Biochemistry 29*, 6179−6186.
Kirilovsky, D., & Etienne, A.-L. (1991) *FEBS Lett. 279*, 201−204.
Kirilovsky, D., Rutherford, A. W., & Etienne, A.-L. (1994) *Biochemistry 33*, 3087−3095.
Klimov, V. V., Dolan, E., Shaw, E. R., & Ke, B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7227−7231.
Koivuniemi, A., Swiezewska, E., Aro, E.-M., Styring, S., & Andersson, B. (1993) *FEBS Lett. 327*, 343−346.
Mac Pherson, A. N., Telfer, A., Barber, J., & Truscott, T. G. (1993) *Biochim. Biophys. Acta 1143*, 301−309.
Michel, H., & Deisenhofer, J. (1986) in *Photosynthesis III: Photosynthetic Membranes and Light-Harvesting Systems. Encyclopedia of Plant Physiology, New Series, Vol. 19* (Staehelin, L. A., & Arntzen, C. J., Eds.) pp 371−381, Springer, Berlin.
Mishra, N. P., Francke, C., van Gorkom, H. J., & Ghanotakis, D. F. (1994) *Biochim. Biophys. Acta 1186*, 81−90.
Petrouleas, V., & Diner, B. A. (1982) *FEBS Lett. 147*, 111−114.
Petrouleas, V., & Diner, B. A. (1990) *Biochim. Biophys. Acta 1015*, 131−140.
Petrouleas, V., Sanakis, Y., Deligiannakis, Y., & Diner, B. A. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) Vol. II, pp 119−122, Kluwer Academic Publ., Dordrecht, The Netherlands.
Petrouleas, V., Deligianakis, Y., & Diner, B. A. (1994) *Biochim. Biophys. Acta 1188*, 271−277.
Richter, M., Ruhle, W., & Wild, A. (1990) *Photosynth. Res. 24*, 229−235.
Sanakis, Y., Petrouleas, V., & Diner, B. A. (1994) *Biochemistry 33*, 9922−9928.
Setlik, I., Allakhverdiev, S. I., Nedbal, L., Setlikova, E., & Klimov, V. V. (1990) *Photosynth. Res. 23*, 39−48.
Styring, S., Virgin, I., Ehrenberg, A., & Andersson, B. (1990) *Biochim. Biophys. Acta 1015*, 269−278.
Theg, S. M., Filar, L. J., & Dilley, R. A. (1986) *Biochim. Biophys. Acta 849*, 104−111.
Trebst, A. (1986) *Z. Naturforsch. 41c*, 240−2445.
van Mieghem, F. J. E., Nitschke, W., Mathis, P., & Rutherford, A. W. (1989) *Biochim. Biophys. Acta 977*, 207−214.
van Wijk, K. J., Andersson, B., & Styring, S. (1992) *Biochim. Biophys. Acta 1100*, 207−215.
Vass, I., & Styring, S. (1993) *Biochemistry 32*, 3334−3341.
Vass, I., Styring, S., Hundal, T., Koivuniemi, A., Aro, E.-M., & Andersson, B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1408−1412.
Vass, I., Gatzen, G., & Holzwarth, A. (1993) *Biochim. Biophys. Acta 1183*, 388−396.
Vermaas, W. F. J., & Rutherford, A. W. (1984) *FEBS Lett. 175*, 243−248.

BI9427882