Influence of DCMU and Ferricyanide on Photodamage in Photosystem II

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ABSTRACT: The effect of strong illumination of thylakoid membranes was studied under a range of conditions. Under anaerobic conditions, the relatively small quenching of the maximum fluorescence (F_{max}) is accompanied by a large increase of the initial fluorescence (F_0), which is partially reversible. Changes in the extent of the $Q_A^-Fe^{2+}$ and chlorophyll triplet EPR signal during anaerobic photoinhibition were consistent with double reduction of Q_A [as reported by Vass et al. ((1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 1408–1412)]. When illumination was done in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or ferricyanide, no change occurred in F_0 while F_{max} was quenched. The quenching of F_{max} occurred more rapidly than the loss of oxygen evolution, and they were both irreversible. In the presence of ferricyanide, the percentage of inhibition of oxygen evolution was larger than the decrease in the extent of the $Q_A^-Fe^{2+}$ signal, indicating that damage of the donor side occurred. In the presence of DCMU, a decrease of the $Q_A^-Fe^{2+}$ EPR signal occurred which corresponded to the inhibition of oxygen evolution and to an increase of the triplet EPR signal, indicating a possible overreduction of Q_A. However, these changes were less marked in the DCMU-treated samples than in the sample without additions and occurred despite the quenching of F_{max}. These results suggest that strong illumination of thylakoids, in the presence of DCMU, results in a slower formation of stable forms of reduced Q_A, thereby allowing the occurrence of side-path reactions leading to F_{max} quenching. From the fluorescence quenching data, these side-path reactions seem to occur under all the conditions studied with the exception of samples illuminated under anaerobic conditions without additions. To rationalize these observations, we propose that, under the latter conditions, relatively rapid overreduction of the reaction center results in the inhibition of charge separation and hence the prevention of F_{max} quencher formation. When overreduction of Q_A is slowed down (by the presence of electron acceptors, oxygen, or DCMU), then side-path reactions occur resulting in the irreversible fluorescence quenching and irreversible damage. These two effects may be related to overoxidation of PS II components.

A decrease of the photosynthetic activity is observed when oxygenic photosynthetic organisms are exposed to high light intensities (photoinhibition). The site of photodamage has been localized at the reaction center of photosystem II (PS II) (for reviews see Andersson & Styring, 1991; Prasil et al., 1992; Aro et al., 1993).

It seems clear that different molecular mechanisms of photoinhibition exist which depend on experimental conditions and biological material. The first site of photodamage depends on the side of PS II where electron transfer becomes a limiting step under high light intensity.[1] Two extreme types of photoinhibition have been well defined *in vitro*: the donor side mediated photoinhibition observed in material where the electron donation to P_{680}^+ is inhibited (Tris-washed, NH_2-OH-treated or Cl^--depleted PS II membranes (Theg et al., 1986; Callahan et al., 1986; Jergerschöld et al., 1990; Blubaugh et al., 1991; Eckert et al., 1991)) and the acceptor side mediated photoinhibition where the removal of electrons from the PS II is the limiting step (thylakoids and PS II membranes in anaerobic or very reducing conditions (Setlik et al., 1990; Kirilovsky & Etienne, 1991; Vass et al., 1992). Related mechanisms were observed during illumination of isolated PS II reaction centers depending on the presence of an electron acceptor or an electron donor (De Las Rivas et al., 1992).

PS II possesses a variable fluorescence emitted by the chlorophyll molecules contained in the protein pigment antenna complex. Two extreme levels of fluorescence can be defined: (1) The initial fluorescence level (F_0), which corresponds to dark adapted cells at the onset of the illumination, represents the fluorescence emitted by PS II centers which are in a photochemical quenching state ready to trap an exciton. (2) The maximal fluorescence level (F_{max}) corresponds to closed centers, generally in the $P_{680}PheoQ_A^-$ state. The rate of fluorescence increase from F_0 to F_{max} is determined by the light intensity and the antenna size. The fluorescence yield can be lowered when some additional quenching occurs in the antenna or in the reaction center. The quenching of fluorescence does not necessarily correspond to a decrease in photochemical yield since the fluorescence yield is such that, even when a maximal light is emitted, it corresponds only to a small percent of the absorbed light. The variable fluorescence ($F_v = F_{max} - F_0$) decreases when a quenching of F_{max} occurs or when the level of F_0 increases. A decrease of variable fluorescence is always observed during photoinhibition (Prasil et al., 1992; Aro et al., 1993).

In the donor side mediated photoinhibition, in PS II membranes in which electron donation from water is inhibited, the electron transfer from Tyr_Z to P_{680}^+ is slowed down with

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[1] Abbreviations: Car, carotenoid; D_1 and D_2, polypeptides of the photosystem II (PS II) reaction center; P_{680}, reaction center chlorophyll (Chl) of PS II; PQ, plastoquinone; Q_A and Q_B, primary and secondary quinone electron acceptor; Ph, pheophytin, the intermediary electron acceptor between P_{680} and Q_A; Tyr_D, the tyrosine 160 of D_2, a side-path electron donor of PS-II; Tyr_Z, the tyrosine 161 of D_1, the electron donor to P_{680}; DMBQ, 2,5-dimethyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; F_0, F_v, F_{max}, initial, variable and maximum fluorescence.

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the simultaneous formation of a quencher of fluorescence (identified as an oxidized carotenoid (Blubaugh et al., 1991)). During a further step, the electron transfer between Tyr_Z and P_{680}^+ is interrupted. The charge separation between P_{680} and Q_A remains functional for a much longer time (Blubaugh et al., 1991; Eckert et al., 1991).

In vitro, in oxygen-evolving material under anaerobic or very reducing conditions, high irradiance leads to a complete reduction of the PQ pool. The variable fluorescence is suppressed during photoinhibition, but F_{max} is quenched to a significantly smaller extent, while F_0 rapidly rises and reaches the F_{max} level (Setlik et al., 1990; Kirilovsky & Etienne, 1991; Vass et al., 1992). The centers are blocked in a high fluorescence state, and oxygen evolution is completely inhibited. The photoinactivation is due to the stabilization of reduced forms of Q_A (Vass et al., 1992, Vass & Styring, 1993). Strong illumination of PS II enriched membranes, under reducing (van Mieghem et al., 1989) and anaerobic (Vass et al., 1992) conditions, led to loss of the EPR signal from $Q_A^-Fe^{2+}$ and the appearance of the long-lived, light-induced chlorophyll triplet state. This was interpreted as a double reduction to form the diamagnetic quinol state in accordance with earlier observations made using strong illumination under reducing conditions (Rutherford & Zimmerman, 1984). Vass & Styring (1993) proposed that an abnormal protonated form of Q_A^- was formed as an intermediate. In this state, the long-lived chlorophyll triplet can be formed (Vass & Styring, 1993).

When the high fluorescence state formed in anaerobic photoinhibition is allowed to relax in the dark, in air at room temperature, the centers slowly reopen, F_0 decreases to low values, and oxygen evolution is partially restored (Kirilovsky and Etienne, 1991). However, some of the blocked centers remain closed (Vass et al., 1992). The relaxation of this high fluorescence state is polyphasic. The $t_{1/2}$ of the different phases varies from seconds to tens of minutes. The slowest decaying phase has been assigned to the reoxidation of Q_AH_2 while the other phases were attributed to the reoxidation of stabilized Q_A^- and of the protonated form of Q_A^- (Vass et al., 1992). The partial irreversibility of the slower phase was proposed to be due to the loss of Q_AH_2 from its site (van Mieghem et al., 1989; Vass et al., 1992). Indeed, it was shown that the binding affinity of the plastoquinone for the Q_A site was lowered after double reduction had occurred (Koivuniemi et al., 1993). The dominant mechanism of photodamage in the presence of oxygen, in oxygen-evolving material, *in vitro* and *in vivo*, is not yet clear. In isolated thylakoids and PS II enriched membranes, high irradiance leads to the formation of a quencher in the PS II which specifically quenches F_{max}. At a somewhat slower rate, the inhibition of the oxygen-evolving activity occurs. Under aerobic conditions, no triplet state could be detected although a decrease of the $Q_A^-Fe^{2+}$ signal was observed (Styring et al., 1990; Vass et al., 1992).

In vivo, a complete reduction of the PQ pool and an accumulation of Q_A^- is less likely than *in vitro* (Krause & Weis, 1991). It may be revelant to consider that in higher plants the PS II is already in a strongly fluorescence quenching down-regulated state when photodamage to the reaction center occurs in high light. In this case, photoinhibition has been reported to occur even when the majority of the PS II centers contain Q_A in its oxidized form (Oquist et al., 1992). Studies on photoinactivation of PS II electron transport and thermoluminescence measurements in *Chlamydomonas reinhardtii* (Ohad et al., 1988) and *Synechocystis* cells (Kirilovsky et al., 1990a) suggested that the initial inhibition was a block in the electron transport between Q_A and Q_B. It was proposed that the photoinactivation of the acceptor side of PS II was followed by inhibition of donor side reactions leading to increased lifetimes of Y_z^+, P_{680}^+, and Chl^+ which might damage D_1 (Gong & Ohad, 1991). In *Synechocystis* cells exposure to high light leads to a quenching of F_{max} with no increase of F_0 (Kirilovsky et al., 1990a). A small increase of F_0 was detected during photoinhibition of *Chlamydomonas* cells (Ohad et al., 1988; Kirilovsky et al., 1990b) or higher plant leaves (Greer et al., 1988). This increase of F_0 is larger when photoinhibition is done under anaerobic conditions (Gong & Ohad, 1991; Godde et al., 1992) but also at low temperature under aerobic conditions (Greer et al., 1988; Kirilovsky et al., 1990b). The high fluorescence state reversed completely to the active low fluorescence state in about 10 min. The decrease of F_0 was hindered by the presence of DCMU and NH_2OH, indicating that the decay of the high level of F_0 corresponded to a slow oxidation of an abnormal stabilized Q_A^- (Kirilovsky et al., 1990b). *In vivo*, the closed centers containing the stabilized Q_A^- seem to be protected from further damage (Kirilovsky et al., 1990b).

The goal of this work is to understand the events leading to the quenched state under aerobic conditions in oxygen-evolving material. For this purpose, we studied two systems in which the presence or absence of oxygen did not change the rate and, apparently, the nature of photodamage: strong illumination of spinach thylakoids in the presence of DCMU or ferricyanide (FeCN). We investigated the relationship between the fluorescence phenomena, the redox states of the reaction center, and the electron flow through the PS II in these processes.

EXPERIMENTAL PROCEDURES

Photoinhibition Experiments. Spinach thylakoids were isolated according to Arntzen et al. (1974), and chlorophyll was determined according to Arnon (1949). Photoinhibitory treatment of spinach thylakoids was performed at 20 °C in a medium containing 50 mM Hepes pH 6.8, 5 mM MgCl, 10 mM NaCl, and 100 mM sorbitol at a chlorophyll concentration of 0.15 mg/mL. The thylakoid suspension (9 mL) was incubated at about 22 °C in a cylindrical glass cell (3-cm diameter) cooled by a water jacket and illuminated by two or four Atralux spots of 150 W (each giving an intensity of about 1000 $\mu E/(m^2 \cdot s)$). The thylakoids were gently stirred by a magnetic bar. Anaerobic conditions were obtained by bubbling argon in the medium for 15 min and then above the sample during the illumination in a closed glass vessel. Samples taken at different times of photoinhibition were stored on ice. The thylakoids photoinhibited in the presence of NH_2OH (3 mM) were preincubated for 5 min with NH_2OH before illumination. When DCMU (10^{-5} M) or ferricyanide (3.3 mM) was used during the photoinhibitory treatment, it was added just before illumination.

Fluorescence Measurements. Fluorescence induction was determined with a fluorimeter described by Vernotte et al. (1982). The fluorescence was excited with a tungsten lamp through a 4-96 Corning filter. The fluorescence was detected in the red region through a 2-64 Corning filter and a Wratten 90 filter. The recording was done through a multichannel analyzer.

The thylakoid samples photoinhibited without additions or in the presence of DCMU were diluted 300-fold to a concentration of 0.5 μg of Chl/mL for the fluorescence measurements. The time between the dilution of the sample and the fluorescence measurement was 1 or 10 min depending on the given experiment (see figure captions for details). When

the measurement was done after 1 min, a high level of F_0 corresponding to a series of postulated reduced forms of Q_A (Q_A^-stable, Q_A•H, Q_AH_2, etc. (Vass et al., 1992) was detected in samples photoinhibited under anaerobic conditions without additions. After 10 min, only the state Q_AH_2 (Vass et al., 1992) was detected.

When NH_2OH and DCMU were present during illumination, the centers were immediately closed in a high fluorescence state corresponding to Q_A^-. This state is stable in the presence of NH_2OH and DCMU. The relaxation of the Q_A^- state formed in the presence of DCMU and NH_2OH upon non-photoinhibitory light (control sample) was completed after sample dilution and 10 min of aerobic, dark incubation. The same time of aerobic incubation was sufficient for a full relaxation of the high fluorescent state in all the photoinhibited samples. Thus, the samples photoinhibited in the presence of DCMU and NH_2OH or only NH_2OH were diluted 300 times and fluorescence measurements were done after 10 min of dark incubation at room temperature.

The thylakoids photoinhibited in the presence of ferricyanide (3.3 mM) were diluted 300 times, and fluorescence measurements were done after 1 min of dark incubation in the presence of DCMU (10^{-5} M).

Oxygen Evolution Measurements. Oxygen evolution of spinach thylakoids (10 μg of Chl/mL) was measured with 2 mM DMBQ, by polarography using a Clark-type oxygen electrode at 25 °C. The samples were illuminated with saturating white light in the presence of two heat filters. The samples photoinhibited in the presence of DCMU were washed three times at 4 °C prior to measuring oxygen evolution. Fluorescence measurements were done before and after the washes. The same rate of decrease of F_v was observed in both measurements. The washes, oxygen evolution, and fluorescence measurements were done with a medium containing 5 mM $MgCl_2$, 10 mM NaCl, 100 mM sorbitol, and 50 mM Hepes, pH 6.8.

EPR Experiments. The amplitudes of the EPR signals from the PS II reaction center triplet and the $Q_A^-Fe^{2+}$ couple were monitored in thylakoids preilluminated with strong light (two lamps) under conditions identical to those used for the fluorescence experiments described above. Following preillumination, the thylakoids were centrifuged and resuspended at high concentration (about 4 mg of Chl/mL) for EPR measurements. Only the long lifetime, high fluorescent state was detected in these samples. The EPR data shown in this article were obtained under anaerobic conditions in the presence of sodium dithionite. Data (not shown) were also taken under anaerobic nonreducing conditions, but as observed earlier (van Mieghem et al., 1992) the triplet state although detectable was much smaller than in dithionite-treated samples. The samples were put into quartz EPR tubes (3-mm i.d.) in the presence of EDTA (1 mM) and dithionite (20 mM). To enhance the $Q_A^-Fe^{2+}$ signal, sodium formate (200 mM) was added to the samples (Vermass & Rutherford, 1984). All samples were bubbled with argon for 5 min and then dark-adapted for 30 min before freezing. EPR spectra of the frozen samples were obtained using a Bruker ESP 300 and Oxford instruments liquid helium cryostat system. Samples were illuminated in the cavity with an 800-W projector to induce the P_{680} triplet signal. EPR settings were as follows: temperature, 4.2 K; modulation amplitude, 20 G; the microwave power was 35 dB (0.05 mW) for the triplet and 8 dB (35 mW) for the semiquinone–iron.

The error in the EPR data taken with thylakoids was quite large, particularly in triplet measurements (about 10%) since the signals were small and were influenced by heating effects. The results shown in Table 1 are the average of three independent experiments for each condition, except that data for photoinhibition in the presence of ferricyanide under aerobic conditions are the result of a single EPR measurement.

Table 1: $Q_A^-Fe^{2+}$ EPR Signal, Triplet Chlorophyll EPR Signal, and Oxygen-Evolving Activity in the Control Sample and in Samples Photoinhibited for 30 min under Different Conditions[a]

treatment	triplet signal (%)	$Q_A^-Fe^{2+}$ signal (%)	O_2 evolution (%)
control	0	100	100
anaerobic	85	15	15
anaerobic + DCMU	40	50	45
anaerobic + FeCN	15	45	25
aerobic	20	45	40
aerobic + DCMU	10	60	54
aerobic + FeCN	20	50	25

[a] 100% of the triplet is calculated on the basis of the assumption that there is an inverse relationship between the presence of the $Q_A^-Fe^{2+}$ and the triplet signal (van Mieghem et al., 1989). In the anaerobic sample, since 15% of the $Q_A^-Fe^{2+}$ signal is still present, it is assumed that 15% of centers are unable to form the triplet state. For this assumption to be correct, it is also required that primary photochemistry ($P_{680}^+Pheo^-$ formation) is not decreased by photodamage in the anaerobic sample without additions.

SDS–Polyacrylamide Gel Electrophoresis and Immunoblotting. The electrophoretic separation of thylakoid polypeptides was performed according to Laemmli (1970) using SDS and 12% acrylamide in the presence of 4 M urea. For immunoblotting experiments, the resolved proteins were transferred to a nitrocellulose filter (Millipore, 0.45 μM) according to Towbin et al. (1979). After overnight incubation at 4 °C with D_1 polyclonal antibody (kindly provided by Prof. I. Ohad), the immunocomplexes were detected by an anti-rabbit secondary antibody alkaline phosphatase conjugate. NBT and BCIP were used as chromogenic substrates for alkaline phosphatase.

RESULTS

We studied the effect of DCMU and ferricyanide on fluorescence quenching, oxygen-evolving inhibition, Q_A^- stabilization, and D_1 degradation during anaerobic and aerobic photoinhibition. The presence of ferricyanide or DCMU during illumination results in contrasting situations regarding the electron transfer: (1) Ferricyanide accepts electrons from Q_A (or/and Q_B) and elsewhere in the electron-transfer chain, greatly increasing the pool of electron acceptors, thus hindering the stabilization of Q_A^-. (2) DCMU inhibits the electron transfer between Q_A and Q_B. In its presence Q_A^- is mainly reoxidized through a back reaction with the positive charges stored on the donor side.

Illumination in the Absence of Oxygen: The Effect of DCMU. Figure 1 shows the effect of strong illumination on the fluorescence and the oxygen evolution activity in thylakoids when the photoinhibitory treatment is performed under anaerobic conditions in the presence and absence of DCMU. As already published (Setlik et al., 1990; Kirilovsky & Etienne, 1991; Vass et al., 1992), in the sample without DCMU, a decrease in F_{max} is accompanied by an increase in F_0 (Figure 1A) while the decrease in F_v closely matches the loss of oxygen evolution (Figure 1B). When DCMU was present during the illumination, the F_{max} level markedly decreased but the F_0 level did not rise (Figure 1A). In addition, the decrease in the F_v level occurred faster than the loss of oxygen evolution (Figure 1B), indicating the presence of centers which have a low, quenched F_{max} but which are capable of oxygen evolution.

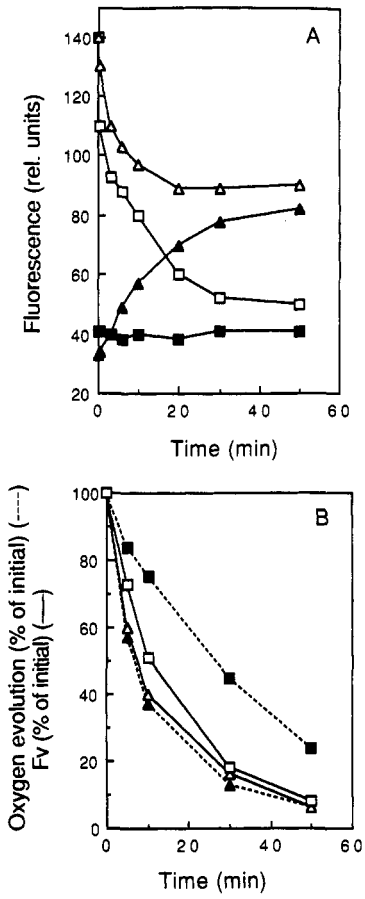

FIGURE 1: Anaerobic photoinhibition in the presence and absence of DCMU. (A) Behavior of F_0 (closed symbols) and F_{max} (open symbols) during anaerobic photoinhibition (four lamps) in the absence (△) or in the presence (□) of 10^{-5} M DCMU. The fluorescence measurements were done 1 min after the dilution in order to detect any high level of F_0. (B) Inhibition of oxygen-evolving activity (closed symbols) and decrease of variable fluorescence (F_v) (open symbols) during anaerobic photoinhibition of spinach thylakoids in the absence (△) or in the presence (□) of DCMU. 100% oxygen evolution in the sample without additions: about 300 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. 100% oxygen evolution in the sample containing DCMU: about 230 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}.

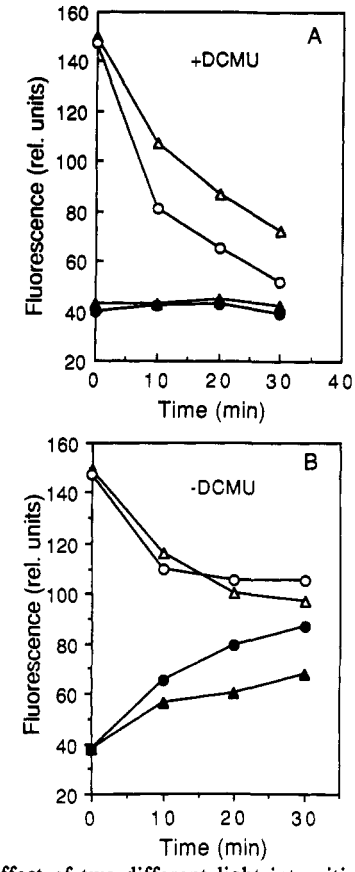

FIGURE 2: Effect of two different light intensities on F_0 (closed symbols) and F_{max} (open symbols) during anaerobic photoinhibition (A) in the presence of DCMU or (B) without additions. The illumination was done by two (△) or four (○) lamps. The fluorescence measurement was done 10 min after dilution in order to detect only the more stable high fluorescent states.

The changes in fluorescence parameters and oxygen rates observed in samples illuminated in the absence of DCMU were partly reversible (about 40–50%) by incubation at room temperature under air (due to reoxidation of overreduced species), while the effects observed in samples illuminated in the presence of DCMU were irreversible under the same conditions. All attempts to reverse the quenching of F_{max} failed. The quenching of F_{max} observed in the sample illuminated in the presence of DCMU could not be reversed by addition of reductants (NH_2OH or ascorbate), oxidants (ferricyanide, DMBQ, DCBQ), or the ionophore gramicidin. These results indicate that the quenching of F_{max} is not due to a simple redox effect nor to an electrochemical gradient. It is also of note that the rate of the decrease in F_{max} was dependent on the photoinhibitory light intensity, in samples in the presence of DCMU, but was independent of light intensity when DCMU was absent (at least at the two intensities tested) (Figure 2). The rate of the increase in the F_0 level in the latter experiment was, however, dependent on light intensity (Figure 2). It seems then that the quenching effects induced by illumination with and without DCMU have different chemical origins.

Low-temperature EPR was used to monitor the redox state of Q_A and the light-inducible reaction center chlorophyll triplet. Spinach thylakoids were photoinhibited, under anaerobic conditions, in the presence or absence of DCMU, for 30 min with two lamps and then concentrated for EPR measurements. The EPR sample without additions was still in a high F_0 level (data not shown). Fluorescence measurements suggested that only the more stable high fluorescence state was present in the EPR sample. In the control sample a large $Q_A^-Fe^{2+}$ EPR signal was present and the Chl triplet signal was absent (Figure 3). After the strong illumination under anaerobic conditions, only 15% of the control $Q_A^-Fe^{2+}$ EPR signal was detected. A large signal from the light-induced P_{680} triplet state (Figure 3A,B) was present in the photoinhibited sample. Since 15% of oxygen-evolving activity remained in this sample (Table 1), we considered that the triplet signal was approximately 85% of the maximal signal. In accord with previous reports (van Mieghem et al., 1989; Vass et al., 1992), we propose that the centers in which the triplet can be generated correspond to centers where the quinone has undergone double reduction. The state $Q_A^{\bullet}H$ has been proposed to be formed as an intermediate prior to double reduction (Vass et al., 1992; Vass & Styring, 1993). Since this state was only detectable at short times of illumination and decays relatively rapidly upon dark adaptation (Vass & Styring, 1993), it is unlikely that it contributes to the EPR signal of the anaerobic photoinhibited sample observed here.

In the sample photoinhibited in the presence of DCMU, which showed a large F_{max} quenching, we also observed a decrease of the $Q_A^-Fe^{2+}$ signal and the appearance of the Chl triplet signal (Figure 3C and Table 1) but to a lesser extent than in the sample photoinhibited without additions. The

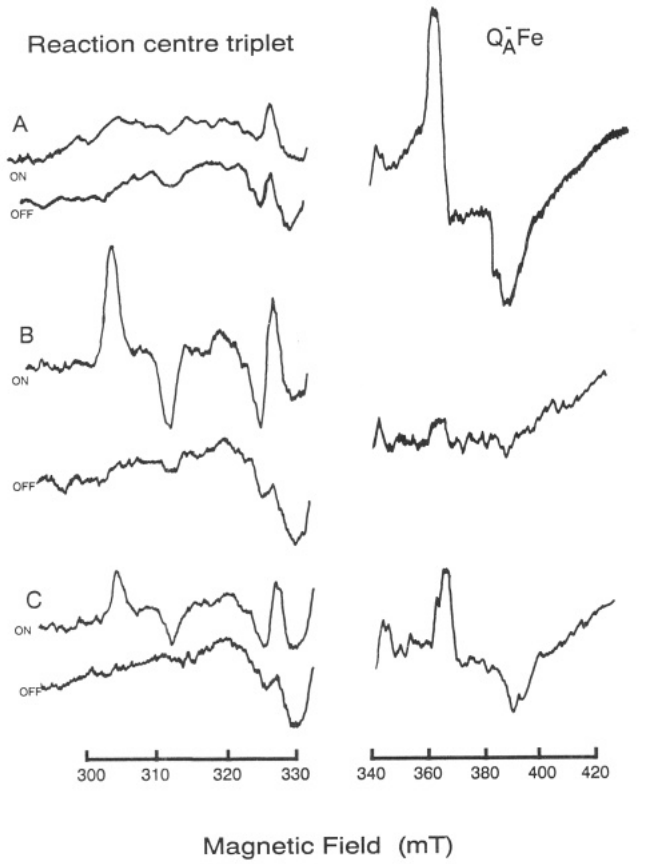

FIGURE 3: Effect of strong illumination on EPR signals arising from PS II. On the left of the figure the reaction center triplet is shown. Spectra were recorded during (ON) and after (OFF) illumination at 4 K. The field range shown covers the low-field half of the triplet spectrum; the high field was omitted because it overlapped with signals arising from the iron sulfur center F_A of PS I, which is photoreduced under these conditions. On the right side of the figure, EPR spectra of $Q_A^-Fe^{2+}$ are shown. (A) Control thylakoids which were not preilluminated. (B) Thylakoids preilluminated under anaerobic conditions. In this sample, only the long-time stable high fluorescence state was detectable. (C) Thylakoids preilluminated under anaerobic conditions but in the presence of 10^{-5} M DCMU. In this sample no increase of F_0 and a large quenching of F_{max} were observed.

chlorophyll triplet was inducible only in 40% of centers. The decrease in the $Q_A^-Fe^{2+}$ signal (50%) corresponded approximately with the loss of activity that occurred as a result of the photodamage (Table 1). Thus, DCMU protects against loss of the $Q_A^-Fe^{2+}$ signal and slows down the appearance of centers in which the Chl triplet was inducible.

The changes observed in the presence or absence of DCMU are unlikely to be due to the actual loss of the protein itself. Figure 4 shows that, under the conditions used in this study, the D_1 protein was not degraded after 50 min of anaerobic photoinhibition. Nedbal et al. (1990) have also demonstrated that, under anaerobic and very reducing conditions, even the very slow photoinactivation of the primary charge separation is faster than protein degradation.

Illumination under Anaerobic Conditions: The Effect of NH_2OH. Strong illumination, under anaerobic conditions, results in the reduction of the plastoquinone pool. After inhibition of the electron transfer between Q_A^- and Q_B, Q_A^- is relatively short-lived in centers having S_2 or S_3 states, while when S_0 or S_1 are present, Q_A^- is long-lived. DCMU is expected to prevent Q_A^- stabilization by preventing formation of $S_0Q_A^-$ or $S_1Q_A^-$ (see Klimov et al. (1985)). To test if DCMU protection from overreduction of Q_A was due to the presence of a reversible S state, the experiments of Figure 1 were

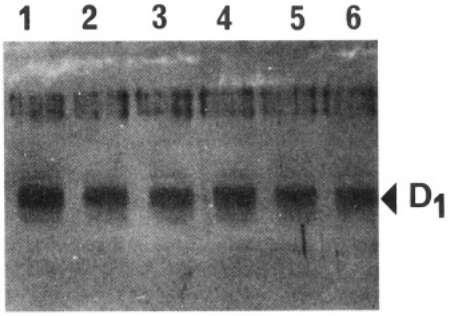

FIGURE 4: D_1 degradation. Immunoblot with D_1 antiserum of control (lanes 1 and 6) and photoinhibited samples for 50 min (with four lamps) without additions (lanes 2 and 3) and in the presence of DCMU (lanes 4 and 5) under aerobic (lanes 2 and 5) and anaerobic (lanes 3 and 4) conditions.

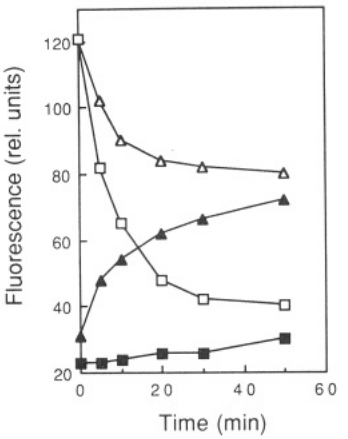

FIGURE 5: F_{max} (open symbols) and F_0 (closed symbols) levels in samples dark incubated with 3 mM NH_2OH for 5 min and then photoinhibited in the presence of NH_2OH as electron donor in the absence (△) or in the presence (□) of DCMU. The fluorescence measurements were done after 10 min of dark incubation of 300-fold-diluted samples.

repeated using thylakoids in which the Mn was removed by NH_2OH treatment and in the presence of NH_2OH as an electron donor.

Figure 5 shows that the fluorescence data in the NH_2OH-treated samples were very similar to those obtained in the oxygen-evolving samples. The high fluorescent states were detected in the samples photoinhibited in the absence of DCMU, while a marked quenching of F_{max} with no F_0 increase was observed in the sample photoinhibited in the presence of DCMU. Thus, the inhibition of the recombination reactions of Q_A^-, by NH_2OH donation, did not impede the irreversible formation of the fluorescence quenching.

Illumination in the Presence of Oxygen: The Effect of DCMU. The effects on fluorescence seen when thylakoids were illuminated under aerobic conditions in the presence and absence of DCMU are shown in Figure 6. In both cases, the level of F_{max} decreased but no increase in F_0 occurred (Figure 6A). In fact, in the presence of DCMU a slight decrease in F_0 was observed (Figure 6A). In both cases, the decrease in F_v was faster than the decrease in oxygen-evolving activity (Figure 6B). Although the decrease in F_{max} was more extensive in the sample illuminated in the presence of DCMU, the rate of photoinhibition of oxygen evolution was almost unaffected by the presence of DCMU. These effects were irreversible. The effects on F_0, F_{max}, F_v, and oxygen evolution seen under aerobic conditions of photoinhibition were similar to those seen under anaerobic photoinhibition conditions in the presence of DCMU.

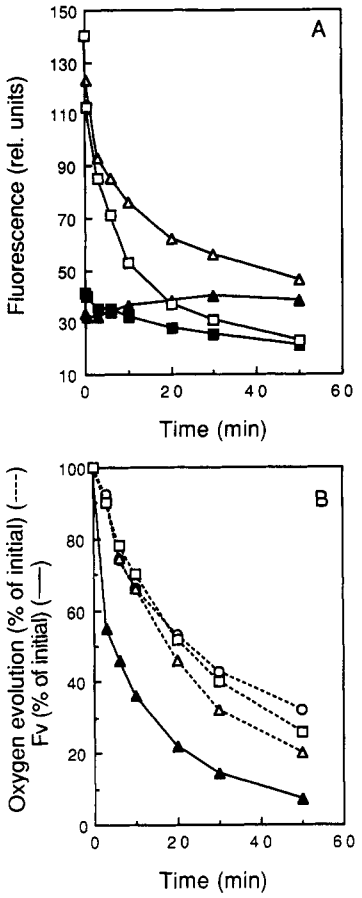

FIGURE 6: (A) Behavior of F_{max} (open symbols) and F_0 (closed symbols) during aerobic photoinhibition in the absence (△) or in the presence (□) of 10^{-5} M DCMU. The fluorescence measurements were done 1 min after the dilution. (B) Inhibition of oxygen evolution during aerobic photoinhibition in the absence (△) or in the presence (□) of DCMU and during anaerobic photoinhibition in the presence of DCMU (○). Also, the F_v decrease during anaerobic photoinhibition is shown (▲). For 100% oxygen-evolving activity of the different samples, see Figure 1.

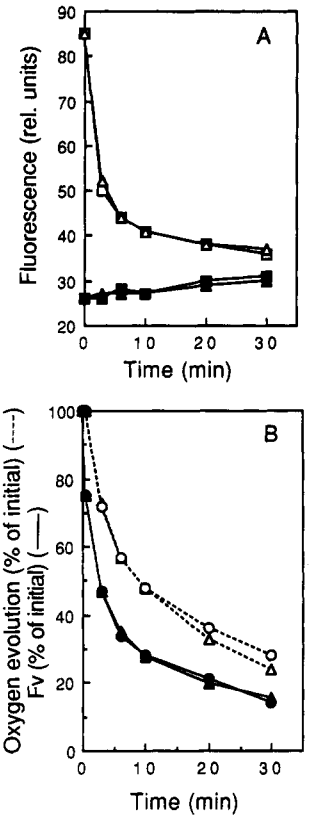

FIGURE 7: (A) F_{max} quenching (open symbols) and F_0 increase (closed symbols) during anaerobic (□) and aerobic (△) photoinhibition in the presence of ferricyanide. The fluorescence measurements were done 1 min after the sample dilution in the presence of 10^{-5} M DCMU. (B) Inhibition of oxygen-evolving activity (open symbols) and the decrease of F_v (closed symbols) during anaerobic (○) and aerobic (△) photoinhibition in the presence of ferricyanide. The oxygen evolution measurements were done as in Figure 1. The thylakoids were photoinhibited with two lamps. 100% of oxygen evolution: about 280 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}.

As reported earlier (Styring et al., 1990; Vass et al., 1992), the EPR spectra taken of samples illuminated under aerobic conditions without additions showed that the $Q_A^-Fe^{2+}$ signal decreased while a small triplet signal was formed (Table 1). DCMU seemed to have little effect under aerobic conditions. Its protection against loss of the $Q_A^-Fe^{2+}$ signal was much less marked than under anaerobic conditions. The decrease in the $Q_A^-Fe^{2+}$ signal corresponded approximately with the loss of oxygen evolution in the presence and in the absence of DCMU (Table 1).

Figure 4 shows that, after 50 min of aerobic illumination in the presence or absence of DCMU, the D_1 protein remained in the membrane. Thus, also under aerobic conditions, the inhibition of the PS II activity precedes the degradation of the protein.

Illumination in the Presence of Ferricyanide. Addition of an exogenous electron acceptor such as FeCN should decrease the stabilization of Q_A^- and thus limit the overreduction of Q_A during strong illumination under anaerobic conditions. Figure 7 shows the fluorescence characteristics and the oxygen-evolving activity in thylakoids when preilluminated in the presence of ferricyanide and in the presence or absence of oxygen.

Under anaerobic conditions, the quenching effects were similar to those seen when DCMU was present: a marked F_{max} quenching with little effect on F_0. In addition, the decay in F_v occurred faster than the loss of the oxygen evolution. We note that the oxygen evolved under these conditions could produce a slight increase in the oxygen concentration despite argon gassing during illumination.

The rates of F_{max} quenching and inhibition of oxygen evolution were unaffected by the absene or presence of oxygen when ferricyanide was present during the illumination (Figure 7). EPR data for thylakoids illuminated in the presence of ferricyanide showed a decrease in the $Q_A^-Fe^{2+}$ signal without a significant increase of the triplet signal (Table 1). The presence of oxygen during the illumination seemed to have little influence on the extent of the two EPR signals (Table 1). In addition, photoinhibition in the presence of ferricyanide gave a situation in which the decrease of the $Q_A^-Fe^{2+}$ signal was less marked than the decrease in the activity (Table 1).

Since it seems less likely that Q_A is overreduced under these conditions, these results indicate that the chemistry occurring is different from that occurring in the anaerobic sample with no additions. Moreover, the results suggest that the photodamage is not accelerated by the presence of oxygen when the stabilization of reduced forms of Q_A is avoided.

DISCUSSION

Strong illumination of spinach thylakoids under anaerobic conditions results in characteristic changes in fluorescence: a large increase in F_0 accompanied by a decrease in F_{max} (Setlik et al., 1990; Kirilovsky & Etienne, 1991; Vass et al., 1992). Quite a different pattern of fluorescence changes (large

quenching of F_{max} without a significant increase in F_0) is observed during strong illumination under a range of contrasting conditions: under anaerobic conditions in the presence of DCMU or ferricyanide; under aerobic conditions with or without ferricyanide or DCMU. Under all of these conditions the decrease of F_v precedes the inhibition of oxygen evolution. By contrast, under anaerobic illumination, the kinetics of the F_v decrease closely matched the kinetics of inhibition of oxygen evolution. The question then arises as to what are the origins of the fluorescence quenching and oxygen evolution inhibition in all of these diverse conditions.

Anaerobic Photoinhibition. Under anaerobic conditions, strong illumination results in the rapid reduction of the electron-transfer chain due to the lack of electron acceptors. After a few turnovers, the plastoquinone pool becomes filled and Q_A is trapped in its semireduced state. Long-lived forms of Q_A^- can be explained by the occurrence of $S_0Q_A^-$ and $S_1Q_A^-$; however, specific stabilizing modifications of Q_A^- may also occur (Setlik et al., 1990; Kirilovsky & Etienne, 1991; Vass et al., 1992). A protonated form of Q_A^- has been proposed to occur as an intermediate in the double reduction of Q_A (Vass et al., 1992; Vass & Styring, 1993). The removal of the negative charge by protonation of Q_A^- (Vass & Styring, 1993), formation of Q_AH_2 (van Mieghem et al., 1989), or perturbation of the center associated with these events results in the appearance of a long-lived chlorophyll triplet upon subsequent radical pair recombination reactions (van Mieghem et al., 1989; Vass & Styring, 1993). The fluorescence levels seen in such samples are attributed to the blockage of forward electron transfer (high F_0) and the increased $P_{680}^+Pheo^-$ radical pair yield (some quenching of F_{max}).

Ferricyanide Effect. When ferricyanide is present, the stabilization of Q_A^- and the overreduction of the acceptor side of PS II will be less likely. Under these conditions, the loss of the $Q_A^-Fe^{2+}$ EPR signal observed did not result in a large increase of the light-inducible chlorophyll triplet and the fluorescence quenching preceded the inhibition of oxygen evolution. In addition, the percentage of inhibition of oxygen evolution was larger than the loss of the $Q_A^-Fe^{2+}$ EPR signal. It seems likely, then, that the fluorescence quenching and the inhibition of oxygen evolution observed here result, at least in part, from a mechanism which is different from that leading to abnormal states of reduced Q_A, possibly involving overoxidation of donor side components. The existence of the small triplet EPR signal may indicate that, despite the presence of ferricyanide, the formation of abnormal states of reduced Q_A also occurred in some centers. The rate and nature of the irreversible photodamage observed in the presence of FeCN were the same under anaerobic or aerobic conditions; thus a major role of oxygen in the photodamage events seems unlikely.

DCMU Effect. When DCMU was present during anaerobic photoinhibition, the decrease in the amplitude of the $Q_A^-Fe^{2+}$ EPR signal, which corresponded to the inhibition of oxygen evolution, matched the increase in the amplitude of the triplet signal. However, the presence of DCMU resulted in a less extensive loss of the $Q_A^-Fe^{2+}$ EPR signal and in a less extensive formation of the triplet signal. In addition, the fluorescence quenching phenomena were like those occurring in samples photoinhibited in the presence of ferricyanide.

In the presence of DCMU, a single photon per center is sufficient to reduce Q_A. Q_A^- can be reoxidized on a time scale of seconds by charge recombination involving oxidized donor side components or is stable for many minutes when NH_2OH is used as an exogenous electron donor. Therefore, subsequent excitations will produce a situation which is similar to that in which Q_A is reduced after filling of the electron acceptor pools. Why, then, does the presence of DCMU give this unexpected photochemistry? We demonstrated that NH_2OH, an exogenous electron donor which displaces the Mn cluster, had no effect on the fluorescence quenching phenomena seen after strong illumination in the presence of DCMU. Thus, the DCMU effect is unrelated to the properties of the S states. An explanation for the DCMU effect could be that conformational changes of the Q_A pocket are involved in the formation of Q_AH_2 (and maybe $Q_A^{\bullet}H$) and that the DCMU binding may hinder such changes. This would allow more charge separation events to occur and hence a greater likelihood of generating the quencher by a side-path reaction before charge separation is switched off by $Pheo^-$ trapping.

There is an alternative, but not exclusive, explanation for the DCMU effect. It has been shown that electrons from the plastoquinol pool can feed into an electron donation cycle involving the cyt b_{559} (Buser et al., 1992). It is likely that the plastoquinone pool is not fully reduced in the DCMU-treated sample. In addition, DCMU blocks reduction of cyt b_{559} by electrons from the plastoquinol pool (Buser et al., 1992). Thus, if such a donation cycle were important in limiting overoxidation of components of PS II (e.g., Chl or carotenoids (Blubaugh et al., 1991; Buser et al., 1992)), the presence of DCMU might actually enhance oxidative events under strong illumination in anaerobic conditions. Thus, irreversible oxidation of side-path components on the donor side could be the origin of the quencher seen under most photoinhibitory conditions. It may be relevant that, although the shape of the Tyr $D^{\bullet}$ EPR signal in the anaerobic sample (without additions) remained the same as that in the control, in all the other samples (i.e., where F_{max} quenching with no F_0 increase occurred), the Tyr $D^{\bullet}$ signal was slightly disturbed by the presence of an extra free radical signal. Although only reflecting a small fraction of the centers in dark-adapted thylakoids, this signal may be related in some way to the existence of the quencher (see, e.g., Blubaugh et al. (1991)).

The fluorescence quenching and inhibition of oxygen evolution occurring during strong illumination in the presence of DCMU were irreversible. From the comparisons between the rates of fluorescence quenching and of the inhibition of oxygen evolution, it seems likely that the quencher formed can be present in the centers which are still active. If the $Q_A^-Fe^{2+}$ EPR signal loss were due to double reduction of Q_A in centers in the presence of the F_{max} quencher, the overreduced Q_A form would appear to be an irreversibly induced damage state.

In the presence of DCMU, the percentage of inhibition of oxygen-evolving activity and that of loss of the $Q_A^-Fe^{2+}$ EPR signal were similar under anaerobic and aerobic photoinhibition. It is significant that, although the triplet EPR signal was smaller in the aerobic sample, the presence or absence of oxygen had no influence on the amount of inactive, low fluorescent centers. This result may indicate that, if singlet oxygen is formed, it damages specifically the centers in which the chlorophyll triplet was formed as proposed by Vass et al. (1992) but that this reaction does not produce an increased rate of irreversible inactivation.

Aerobic Photoinhibition, in Vitro. Strong illumination of thylakoids under aerobic conditions has been suggested to produce overreduction of Q_A leading to triplet formation (Styring et al., 1990), just as seen under anaerobic conditions (Vass et al., 1992). We observed, in agreement with earlier work (Styring et al., 1990), that aerobic illumination resulted in a decrease in the $Q_A^-Fe^{2+}$ EPR signal and in the formation

of very little triplet signal. The suggestion that the triplet signal is small because of a reaction with oxygen leading to the loss of photochemistry (Vass et al., 1992) is an adequate explanation of the EPR phenomena presented in their paper. However, it has been shown that, during aerobic photoinhibition of spinach thylakoids or of PS II-enriched membranes, centers in which no $Q_A^-Fe^{2+}$ EPR signal could be induced still had the capacity to form the primary radical pair $P_{680}^+Pheo^-$ as measured by photoaccumulation of $Pheo^-$ (Allakhverdiev et al., 1987; Styring et al., 1990; van Wijk et al., 1992). No triplet signal was detected in these centers (Styring et al., 1990; van Wijk et al., 1992). This inconsistency may indicate that loss of the $Q_A^-Fe^{2+}$ EPR signal may not be associated with overreduction of Q_A in aerobic photoinhibition. Changes in the environment of Q_A or modifications in the properties of the Fe^{2+} may also produce a loss of the $Q_A^-Fe^{2+}$ EPR signal. Such changes could conceivably be triggered by events elsewhere in the reaction center.

We showed that the fluorescence changes observed in aerobic photoinhibition were similar to those observed when thylakoids were illuminated in the presence of an electron acceptor or in the presence of DCMU. The events occurring under aerobic conditions may be rationalized by assuming that there is a continual electron flow to oxygen via the Mehler reaction. It is to be expected that oxygen is a less efficient acceptor than ferricyanide and overreduction of PS II may still occur. However, it seems likely that stabilization of reduced forms and double reduction of Q_A are at least slowed down compared to anaerobic conditions, and thus the possibility is allowed for different photochemistry to occur leading to irreversible changes: quencher formation and inhibition of oxygen evolution. Thus we consider that photodamage occurring under aerobic conditions may involve a mechanism other than double reduction of Q_A. The overoxidation of the donor side of PS II (perhaps chlorophyll or carotenoid; see, e.g., Blubaugh et al. (1991)) may be involved in quencher formation. Styring et al. (1990) observed that during aerobic photoinhibition the cyt b_{559} became oxidized at early times. An accumulation of oxidized Chl might be expected under these circumstances and could specifically quench F_{max}.

Aerobic Photoinhibition, in Vivo. It is worth considering the relevance of the phenomena observed *in vitro* to photoinhibition occurring *in vivo*. Strong illumination of *C. reinhardtii* cells under aerobic conditions results in two very different behavior for fluorescence and oxygen evolution depending only on the temperature at which photoinhibition is performed (Kirilovsky et al., 1990b). At 5 °C, it results in slower reoxidation of the electron acceptor side of PS II, hence in the faster accumulation of reduced Q_A, resulting in inhibition of PS II electron transfer. Stabilized forms of Q_A^- were formed. Despite the presence of oxygen during the strong illumination, the inactivation was 100% reversible (Kirilovsky et al., 1990b), indicating that no damage occurred to the centers. These results suggests that the formation of Chl triplet inducing states was very unlikely. When the same photoinhibitory treatment was done at 25 °C, it produced an irreversible inhibition of oxygen evolution, accompanied by a large F_{max} quenching (Kirilovsky et al., 1990b). Since electron transfer out of PS II is no longer slowed down, formation of $Q_A^{\cdot}H$ or overreduction of Q_A seems less likely under these conditions than at 5 °C. The same type of results were obtained in whole leaf photoinhibition (Greer et al., 1988). Moreover, Gong & Ohad (1991) observed that the degradation of D_1 was slower in mutants deficient in plastoquinol oxidation than in wild-type *C. reinhardtii*. Therefore, *in vivo*, the link between photodamage, stabilization of reduced Q_A, triplet inducing states, and production of singlet oxygen is not evident. However, it has recently been suggested that, under weak light, chlorophyll triplet/singlet oxygen mediated protein damage may occur through charge recombination reactions of PS II (the oxidized S_2 and S_3 states with the reduced acceptor side, Q_A^- or Q_B^-) (Keren et al., 1993).

CONCLUSIONS

In the present work, we studied irreversible photoinhibition in the presence of DCMU and ferricyanide and we have shown that their fluorescence and EPR characteristics are very similar to those seen in aerobic samples with and without additions. Moreover, the rates of inhibition of oxygen evolution were very close under aerobic or anaerobic illumination when DCMU or ferricyanide was present. We suggest that, under strong illumination, light-induced charge separation gives rise to a low quantum yield side reaction which generates a quencher. When thylakoids are anaerobically illuminated without additions, this side reaction occurs to a lesser extent than in all the other samples tested, because overreduction of Q_A takes place efficiently leading to $Pheo^-Q_AH_2$ formation, a state in which no further charge separation takes place. In all the other conditions, this state is more difficult to trap; therefore, more charge separations and, hence, more F_{max} quencher formation occur. This fluorescence quencher, which specifically quenches F_{max} and dominates the fluorescence phenomenology under most photoinhibitory conditions (with the exception of anaerobic without additions), might be associated with formation of Chl^+ or Car^+ cations in the reaction center as is thought to occur in centers in which electron transfer from water is inhibited prior to illumination (Blubaugh et al., 1991). The formation of the quencher seems, in itself, not to result in inhibition of oxygen evolution, but it is an irreversible event. Moreover, its existence seems not to prevent further photodamage, which, in the presence of the quencher, becomes irreversible.

We propose that under aerobic conditions (without additions), *in vitro* and *in vivo*, the photoinhibition of oxygen evolution results from the combination of modifications of the acceptor side of PS II, overreduction of Q_A, and overoxidation of the donor side. The balance between these mechanisms will depend on the relative efficiency of electron donor side and electron acceptor side reactions in PS II.

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REFERENCES

Allakhverdiev, S. I., Setlikova, E., Klimov, V. V., & Setlik, I. (1987) *FEBS Lett. 226*, 186–190.

Andersson, B., & Styring, S. (1991) in *Current Topics in Bioenergetics* (Lee, C. P., Ed.) Vol. 16, pp 1–81, Academic Press, New York.

Arnon, D. I. (1949) *Plant Physiol. 24*, 1–15.

Arntzen, C. J., Vernotte, C., Briantais, J.-M., & Armond, P. (1974) *Biochim. Biophys. Acta 368*, 39–53.

Aro, E.-M., Virgin, I., & Andersson, B. (1993) *Biochim. Biophys. Acta 1143*, 113–134.

Blubaugh, D. J., Atamian, M., Babcock, G. T., Golbeck, J. H., & Cheniae, G. M. (1991) *Biochemistry 30*, 7586–7597.

Buser, C. A., Diner, B. A., & Brudvig, G. W. (1992) *Biochemistry 31*, 11449–11459.

Callahan, F. E., Becker, D. W., & Cheniae, G. M. (1986) *Plant Physiol. 82*, 261–269.
De Las Rivas, J., Andersson, B., & Barber, J. (1992) *FEBS Lett. 301*, 246–252.
Eckert, H.-J., Geiken, B., Bernarding, J., Napiwotzki, A., Eichler, H.-J., & Renger, G. (1991) *Photosynth. Res. 27*, 97–108.
Godde, D., Buchold, J., Ebbert, V., & Ottmeier, W. (1992) *Biochim. Biophys. Acta 1140*, 69–77.
Gong, H., & Ohad, I. (1991) *J. Biol. Chem. 266*, 21293–21299.
Greer, D. H., Laing, W. A., & Kipnis, T. (1988) *Planta 174*, 152–158.
Jergerschöld, C., Virgin, I., & Styring, S. (1990) *Biochemistry 29*, 6179–6186.
Keren, N., Gong, H., & Ohad, I. (1993) in *Abstracts from the 41st Harden Conference: Photoinhibition of Photosynthesis: from Molecular Mechanisms to the Field*, p 51, Biochemical Society, London.
Kirilovsky, D., & Etienne, A.-L. (1991) *FEBS Lett. 279*, 201–204.
Kirilovsky, D, Ducruet, J.-M., & Etienne, A.-L. (1990a) *Biochim. Biophys. Acta 1020*, 87–93.
Kirilovsky, D., Vernotte, C., & Etienne, A.-L. (1990b) *Biochemistry 29*, 8100–8106.
Klimov, V. V., Shuvalov, V. A., & Heber, U. (1985) *Biochim. Biophys. Acta 809*, 345–350.
Koivuniemi, A., Swiezewska, E., Aro, E.-M., Styring, S., & Andersson, B. (1993) *FEBS Lett. 327*, 343–346.
Krause, G. H., & Weis, E. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 42*, 313–349.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Nedbal, L., Masojidek, J., Komenda, J., Prasil, O., & Setlik, I. (1990) *Photosynth. Res. 24*, 89–97.
Ohad, I., Koike, H., Shochat, S., & Inoue, Y. (1988) *Biochim. Biophys. Acta 933*, 288–298.
Oquist, G., Chow, W. S., & Anderson, J. M. (1992) *Planta 186*, 450–460.
Prasil, O., Adir, N., & Ohad, I. (1992) in *The Photosystems, Topics in Photosynthesis* (Barber, J., Ed.) Vol. 11, pp 220–250, Elsevier, London.
Rutherford, A. W., & Zimmermann, J. L. (1984) *Biochim. Biophys. Acta 767*, 168–175.
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.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4353.
van Mieghem, F. J. E., Nitschke, W., Mathis, P., & Rutherford, A. W. (1989) *Biochim. Biophys. Acta 977*, 207–214.
van Mieghem, F. J. E., Searle, G. F. W., Rutherford, A. W., & Schaafsma, T. J. (1992) *Biochim. Biophys. Acta 1100*, 198–206.
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., Hundall, T., Koivuniemi, A., Aro, E.-M., & Andersson, B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1408–1412.
Vermass, W. F. J., & Rutherford, A. W. (1984) *FEBS Lett. 175*, 243–248.
Vernotte, C., Etienne, A.-L., & Briantais, J.-M. (1982) *Biochim. Biophys. Acta 545*, 519–527.