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Strong light photoinhibition of electrontransport in Photosystem II. Impairment of the function of the first quinone acceptor, Q_A

Stenbjörn Styring [1], Ivar Virgin [1], Anders Ehrenberg [2] and Bertil Andersson [1]

[1] *Department of Biochemistry and* [2] *Department of Biophysics, Arrhenius Laboratories for Natural Sciences, University of Stockholm, Stockholm (Sweden)*

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Electron paramagnetic resonance (EPR) spectroscopy has been applied in an investigation on the mechanism for photoinhibition of the electron transport in Photosystem II. The experiments were performed in vitro in thylakoid membranes and preparations of Photosystem-II-enriched membranes. Photoinhibition resulted in inhibition of the oxygen evolution and EPR measurements of the S_2 state multiline EPR signal show that its induction by illumination at 198 K was decreased with the same kinetics as the oxygen evolution. Further EPR measurements show that the reduction of Q_A was inhibited with the same kinetics as the oxygen evolution. The amount of photoreducible pheophytin was estimated from photoaccumulation experiments under reducing conditions and the results show that the primary charge separation reaction was inhibited much slower than the oxygen evolution or the reduction of Q_A. These results indicate that photoinhibition inhibits the electron transfer between pheophytin and Q_A probably by impairment of the function of Q_A. In the inhibited centers the primary charge separation reaction is still operational. It is suggested that the event leading to photoinhibition of the electron transport is the double reduction of Q_A which then leaves its site. Photoinhibition also results in rapid oxidation of cytochrome *b*-559 and a change of cytochrome *b*-559 from its high potential form to its low potential form. The reaction is quantitative and proceeds with the same kinetics as the inhibition of oxygen evolution. The potential shift of cytochrome *b*-559 suggests that photoinhibition induces early conformational changes in Photosystem II.

Introduction

Strong illumination of oxygenic photosynthetic organisms results in decreased CO_2 fixation, inhibition of photosynthetic electron transport and oxygen evolution [1]. There is now a general agreement that Photosystem II is the primary target for the photoinhibitory process [2]. The electron transfer reactions in Photosystem II are complex (for reviews see Refs. 3,4) and it is not clear at what particular step and by which molecular mechanism the photoinhibition occurs.

In the reaction center of Photosystem II the absorbtion of a light quantum results in a fast primary charge separation between the primary electron donor P680 and the intermediary electron acceptor, a pheophytin molecule. From pheophytin the electron is rapidly transferred to the enzyme-bound primary quinone acceptor, Q_A. Under normal conditions, Q_A only accepts one electron, which is transferred to Q_B, the second plastoquinone acceptor. Q_B which can accept two electrons, exchanges with the plastoquinone pool when it is fully reduced, but remains bound to the protein in its semi-reduced form [5,6]. A ferrous iron is situated midway between the Q_A and Q_B molecules [3]. The oxidized $P680^+$ is rapidly reduced by a tyrosine residue (Tyr_Z) [7] which, in its turn, is reduced by the oxygen evolving complex. This is composed of a Mn cluster containing 2–4 Mn atoms [8], and at least partially the oxidizing equivalents needed to oxidize water are stored on the Mn ions [9,10]. Tyr_Z, P680, pheophytin, Q_A, Q_B and Tyr_D (a sidepath electron donor [11,12]) are associated with the heterodimer of the D1 and D2 proteins which comprises the reaction center of Photosystem II [13]. The location of the Mn cluster is at present less clear,

Abbreviations: Mes, 4-morpholineethanesulphonic acid; P680, the primary electron donor; Q_A, the first quinone acceptor; Q_B, the second quinone acceptor, Tyr_Z, tyrosine residue functioning as the immediate electron donor to $P680^+$.

Correspondence: S. Styring, Department of Biochemistry, Arrhenius Laboratories for Natural Sciences, University of Stockholm, S-10691 Stockholm, Sweden.

but recent evidence sugests that this also is bound to the D1 protein [10,14,15].

Photoinhibition not only results in inhibition of the electron transport through Photosystem II but it also leads to degradation of the D1 and (to a smaller extent) the D2 proteins. In vivo this is observed as a rapid, light dependent turnover of the D1 protein which is accelerated by stress conditions known to lead to photoinhibition [14,16–19]. The observation that the reaction center protein D1 is sensitive to strong light is intriguing and has led to considerable efforts to deduce which reactions initiate photoinhibition and damage to the protein. Essentially two schools have developed. The first suggests that electron transfer is inhibited at the level of Q_B and that the primary damage leading to degradation of the D1 protein is initiated at the Q_B site [16,17,20], while the second school implies that one or other of the primary reactions in Photosystem II between Tyr_Z and Q_A is inhibited [21–24].

Here we have applied EPR spectroscopy in an attempt to resolve some of the questions concerning the reactions leading to photoinhibition and damage to Photosystem II. The advantage of EPR spectroscopy is that it permits direct observation of most of the redox components in Photosystem II using illumination at various temperatures of nontreated or chemically reduced samples. With this technique we show that photoinhibition of isolated thylakoid membranes or Photosystem II enriched membranes leads primarily to impairment of the electron acceptor function of Q_A. In spite of this inhibition, the primary charge separation reaction is still operational. Preliminary accounts of this work have been given earlier [25].

Materials and Methods

Preparations of thylakoid membranes and Photosystem-II-enriched membranes

Thylakoid membranes isolated from spinach leaves as in Ref. 26 were either used directly or frozen in liquid nitrogen at high chlorophyll concentration in 5% dimethylsulfoxide. Photosystem-II-enriched membranes were prepared as in Ref. 27. The isolated membranes were suspended to 10 mg chlorophyll/ml in 20 mM Mes-NaOH (pH 6.3), 15 mM NaCl and 0.4 M sucrose and stored in liquid nitrogen.

Photoinhibition of photosynthetic membranes

Photoinhibition was carried out aerobically at 20 °C. Thylakoid membranes were diluted to 150 μg chlorophyll/ml in 10 mM sodium phosphate (pH 7.4) containing 5 mM NaCl, 5 mM $MgCl_2$ and 100 mM sucrose and exposed to white light (approx. 5000 $\mu E/m^2$ per s) for various times. Photoinhibition of Photosystem-II-enriched membranes (100–200 μg chlorophyll/ml) was performed aerobically at 20 °C in the suspension buffer using the same light intensity. After the illumination procedure the membranes were collected by centrifugation and resuspended to 6 mg chlorophyll/ml (thylakoid membranes) or 4–5 mg chlorophyll/ml (Photosystem II enriched membranes). Samples intended for EPR measurements were transferred to calibrated EPR tubes and illuminated by room light for 15 s. Before freezing, the samples were incubated in the dark for 15 min to allow recombination of any reduced electron acceptors.

Oxygen evolution measurements

Photosystem-II-mediated oxygen evolution was measured polarographically at saturating light using phenyl-*p*-benzoquinone as exogenous electron acceptor. The assay medium for thylakoid membranes was composed of 30 mM phosphate buffer (pH 6.5), 3 mM NaCl, 60 mM sucrose, 0.4 mM phenyl-*p*-benzoquinone and for Photosystem II enriched membranes 20 mM Mes-NaOH (pH 6.3), 10 mM NaCl, 5 mM $CaCl_2$, 100 mM sucrose and 0.4 mM phenyl-*p*-benzoquinone. The oxygen evolution measurements were performed with samples taken from the prepared EPR samples immediately before the freezing to allow direct comparison of oxygen evolution with measured EPR characteristics.

EPR spectroscopy

X-band low-temperature EPR spectra were recorded at a microwave frequency of 9.239 GHz with a Bruker ESP 300 spectrometer using the ESP 300 program or at 9.39 GHz with a Bruker ER 200 D-SRC spectrometer equipped with an Aspect 2000 computer. Both spectrometers were equipped with an Oxford Instruments cryostat and temperature controller. Normalization of EPR spectra for tube-calibration factors and chlorophyll concentrations were done with the computer.

Sodium formate was added from a 0.5 M solution adjusted to the appropriate pH with formic acid. Dithionite was added from an anaerobic 0.5 M solution in the appropriate buffer solution. Incubation with formate (25 mM) and dithionite (50 mM) was performed at 20 °C, in the dark, for 10 min.

Illumination of EPR samples was done in an unsilvered dewar from a 1000 W projector lamp (through heat-absorbing $CuSO_4$ and a heat-reflecting filter). In order to perform one single stable charge separation, illumination of unreduced samples was done for 4 min at 198 K (a solid CO_2/ethanol slurry). In experiments intended to phototrap reduced pheophytin, illumination of dithionite reduced samples was performed for 40 min at 198 K or 25 min at 293 K.

Quantification of oxidized cytochrome *b*-559 was done by single peak integration using the spectral turning point around $g = 3$ according to Aasa and Vänngård [28]. The radical EPR signal II_{slow} was integrated using the same method. For spin-quantification on a reaction

center basis we compared cytochrome *b*-559 with Signal II_{slow}. This signal originates from the cationic radical of Tyr-160 [11,12] in the D2 protein and amounts to, in a shortly dark-incubated control sample, one radical/Photosystem II reaction center [11,12,29].

Results

Effect of photoinhibition on electron donors

Strong illumination at 20 °C of thylakoid or Photosystem-II-enriched membranes progressively inhibited the steady-state electron-transport from water to the electron acceptor (Fig. 1A, 2A). We have applied EPR spectroscopy to correlate the impairment of the function of different redox components in Photosystem II with the inhibition of the overall electron transport. The results show that the formation of the multiline EPR signal, originating from the S_2 state, was inhibited in correlation with the inhibition of the photosynthetic electron transport both in photoinhibited thylakoid membranes [14] and Photosystem-II-enriched membranes (Fig. 2A,B). In the experiments, the S_2 state multiline EPR signal was induced by illumination at 198 K of the EPR samples prepared from the photoinhibited membranes. At this temperature, the illumination leads to the quantitative reduction of Q_A but the electron transfer to Q_B is blocked [30]. Thus, only one electron is taken from the donor side of Photosystem II. In dark-incubated material, (which has the oxygen evolving complex mainly in the S_1-state [31]), the S_1 to S_2 transition dominates [32–34] leading to the formation of the S_2 state multiline EPR signal [35,36]. In centers where this transition for some reason can not occur, electron donation from cytochrome *b*-559 or a chlorophyll molecule is observed [33]. Both of these oxidized species can be quantified with EPR spectroscopy and we have measured both these putative electron donors. The results show that photoinhibition, did not increase the oxidation of cytochrome *b*-559 induced by the secondary illumination at 198 K either in thylakoid membranes (Fig. 1B) or in Photosystem-II-enriched membranes (not shown). The observed light induced oxidation of the high potential form of cytochrome *b*-559 in the dark control (Fig. 1B, the upper pair of spectra) is

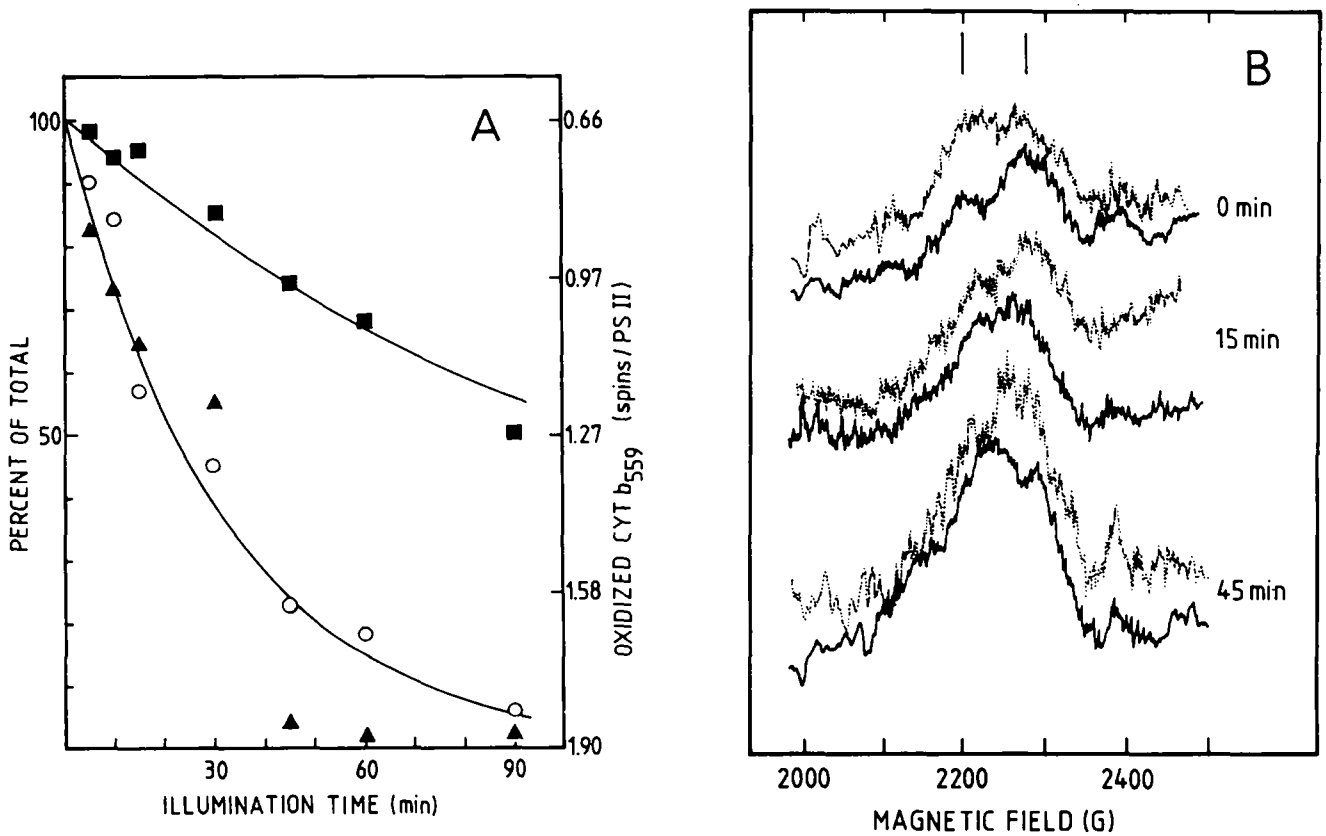


Fig. 1(A) The effect of strong light (≈ 5000 $\mu E/m^2$ per s) photoinhibition of thylakoid membranes on the oxygen evolution (circles), the amount of oxidized cytochrome *b*-559 (triangles) and the amount of the D1 protein (squares). The curves represent single exponential fits of the data for the oxygen evolution and the degradation of the D1 protein assuming half decay times of 21 and 106 min, respectively. (B) EPR spectra of the g_z region of oxidized cytochrome *b*-559 recorded after various times of photoinhibition of thylakoid membranes. Full-line spectra were recorded after 15 min dark incubation of the photoinhibited samples. The dotted spectra were recorded after 4 min continuous illumination at 198 K. The bars indicate the position for the $g = 3.06$ peak (at 2200 G) and the $g = 2.94$ peak (at 2270 G). EPR conditions: $T = 15$ K; microwave power, 15 dB below 200 mW; modulation amplitude, 12.5 G; microwave frequency, 9.39 GHz.

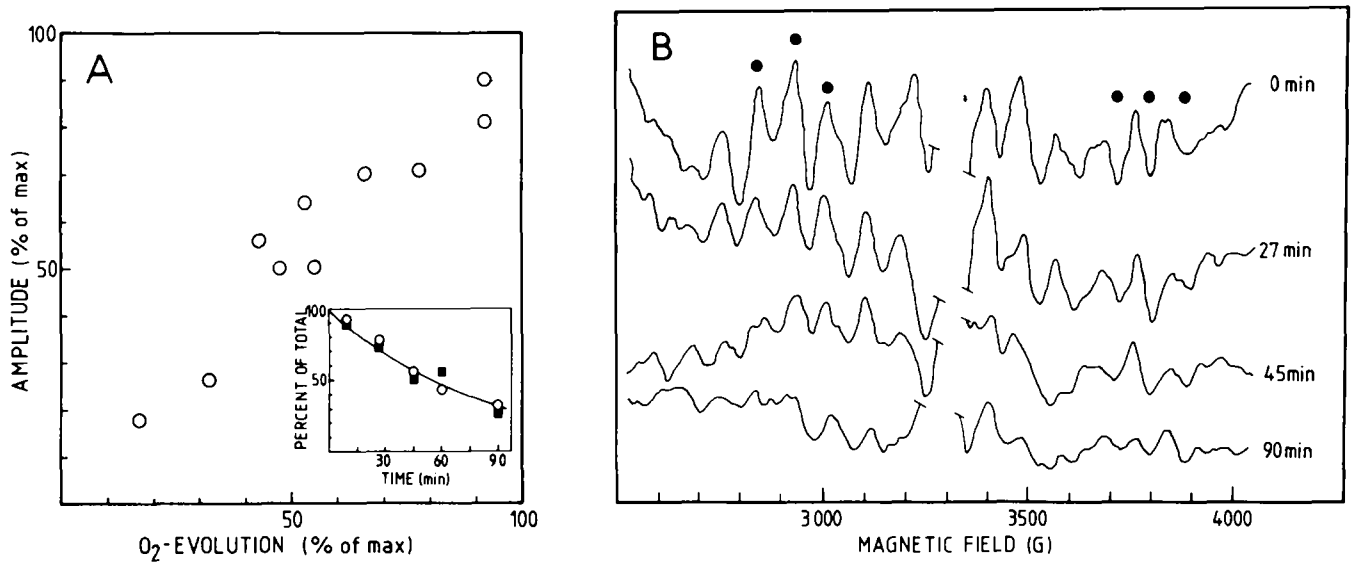


Fig. 2(A) Correlation between the inhibition of oxygen evolution and the diminished formation of the S_2-state multiline EPR signal (induced by 198 K illumination) as a consequence of photoinhibition of Photosystem II enriched membranes. The inset shows the time-course for the loss of the oxygen evolution (open circles) and the multiline signal formation (squares) in Photosystem-II-enriched membranes. The curve represents a single exponential fit to the data assuming a half-time for the inhibition of 52 min. (B) Decrease of the S_2-state multiline signal as a consequence of photoinhibition for various times of Photosystem-II-enriched membranes. The EPR spectra show the signals induced by illumination at 198 K. The added peak heights of the indicated peaks were used as a measure of the signal size. The amplitude in the dark sample (0 min) was set as 100% signal in Fig. 2A. EPR conditions: Temperature 10 K; microwave power 10 dB down from 200 mW; modulation amplitude 22 G; microwave frequency 9.239 GHz.

normal and partly due to centers that were in the S_0 state after the dark incubation. Here it must be pointed out that photoinhibition resulted in oxidation of cytochrome *b*-559 (Fig. 1B) before the secondary illumination at 198 K (see below). In addition, the formation of the chlorophyll radical was investigated and the yield of chlorophyll$^+$ induced by illumination at 198 K gradually decreased as a consequence of photoinhibition (not shown).

Thus, photoinhibition results in a gradual decrease in low temperature electron donation to $P680^+$ from the known electron donors in Photosystem II, i.e., the Mn cluster, a chlorophyll molecule and cytochrome *b*-559. The decrease in the electron donation closely follows the inhibition of oxygen evolution in both thylakoid membranes and Photosystem-II-enriched membranes.

Effects of photoinhibition on the reduction of Q_A

With EPR spectroscopy the reduced first quinone acceptor Q_A can be measured. The observable spectrum originates from magnetic interactions between the reduced quinone, Q_A^-, and the nearby situated ferrous iron [37]. The spectrum is difficult to measure in thylakoid membranes but the signal (at $g = 1.82$) is easier to observe in Photosystem-II-enriched membranes [38]. In addition, its size can be further enhanced more than 10-fold by the addition of formate [39]. This technique was applied in an experiment where Photosystem-II-enriched membranes were photoinhibited for various times. In the EPR samples that were prepared from the photoinhibited material Q_A was reduced, in the presence of formate, either photochemically by illumination at 198 K or chemically using dithionite. Fig. 3A shows the chemically induced spectra and it is seen that the signal size progressively is diminished during photoinhibition. The decrease correlates with the inhibition of oxygen evolution (Fig. 3C,D). Also the photochemical reduction of Q_A was inhibited with the same kinetics as oxygen evolution (not shown).

The effects of photoinhibition on the intermediary electron acceptor pheophytin

Another spectroscopic probe to the integrity of the acceptor side complex in Photosystem II is the so called 'split pheophytin' EPR signal [40,41] which originates from magnetic interactions between the pheophytin$^-$ and the reduced Q_A^--Fe^{2+} complex and can be observed when both pheophytin and Q_A are reduced. The EPR signal is centered around $g = 2$ and characterized by a splitting of 30–35 G in the presence of formate [39]. Photoaccumulation of pheophytin$^-$ in the presence of Q_A^- is achieved by strong illumination at 198 K in the presence of dithionite [41].

The 'split pheophytin' EPR signal was generated in samples from an experiment in which Photosystem-II-enriched membranes were photoinhibited for various times. The results (Fig. 3B–D) show that the signal decreases in size in correlation with the inhibition of the oxygen evolution. The EPR signal is observable only in centers where pheophytin and Q_A are reduced simulta-

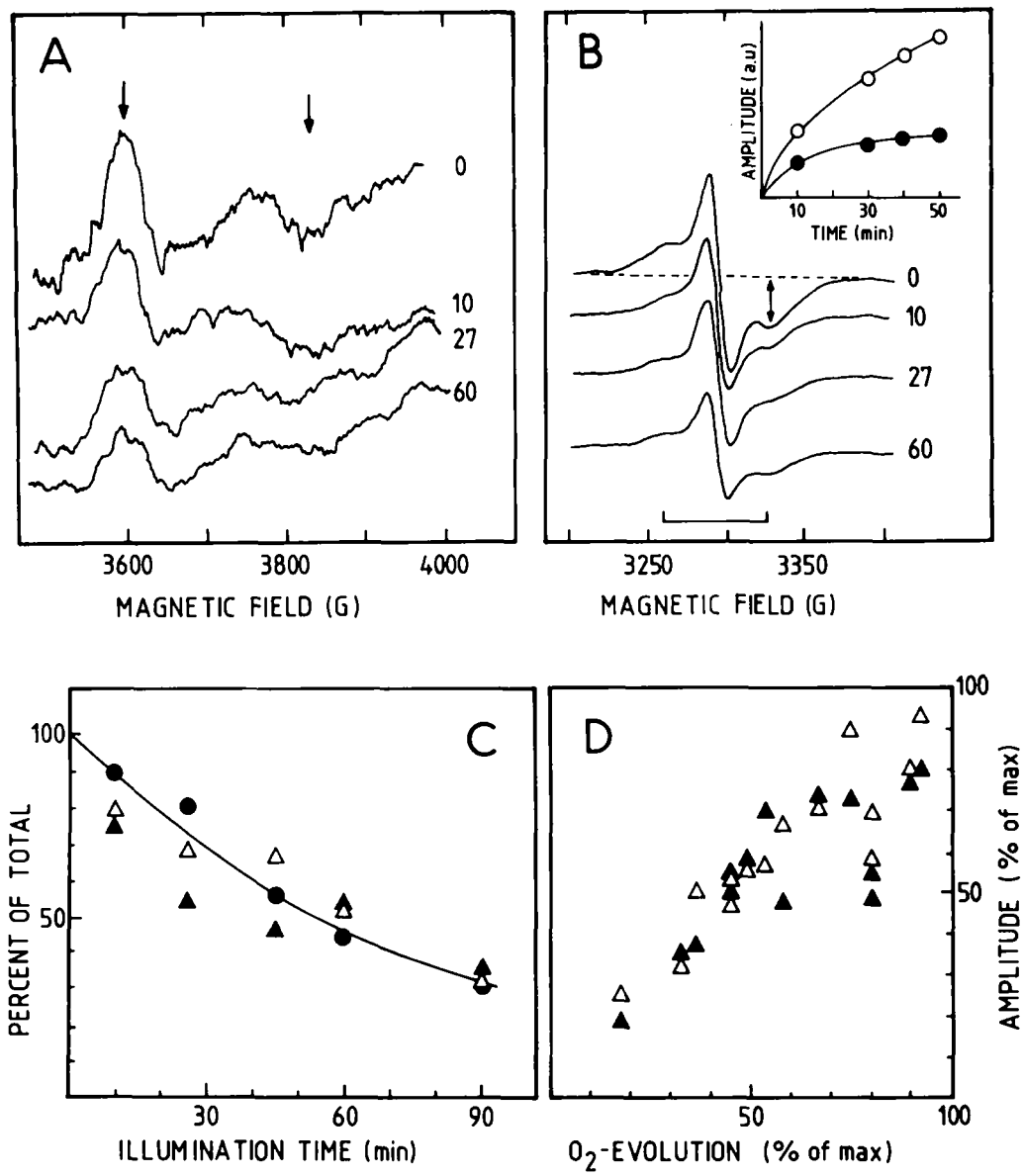


Fig. 3(A) The effect of photoinhibition on the formation of the EPR spectrum from the reduced Q_A^--Fe^{2+} signal in Photosystem-II-enriched membranes. Q_A was reduced chemically by dithionite (50 mM) in dark-adapted samples exposed to various times of photoinhibitory illumination (0, 10, 27 and 60 min). The spectra are recorded in the presence of 25 mM formate to enhance the signal size. The arrows indicate the spectral features at $g = 1.82$ (at 3600 G) and $g \approx 1.7$ (around 3830 G) that belong to the signal. EPR conditions: temperature, 4 K; microwave power 8 dB down from 200 mW; modulation amplitude 32 G; microwave frequency 9.239 GHz. (B) The effect of photoinhibition on the formation of the 'split pheophytin' signal. The samples are the same as in Fig. 3A except that they have been illuminated for 40 min at 198 K in the presence of dithionite and formate. The bars indicate the field position for the split pheophytin lines reported in the presence of formate [39]. The arrow marks the field chosen for the measurement of the amplitude of the signal. The inset shows the light saturation at 198 K of the split signal (closed circles) and the center peak structure (open circles). EPR conditions as in (A), except for the modulation amplitude, which was 10 G. (C) The effect of photoinhibition of Photosystem-II-enriched membranes on the oxygen evolution (filled circles), the formation of the Q_A^--Fe^{2+} EPR signal (open triangles) and the formation of the 'split pheophytin signal' (filled triangles). The amplitude at $g = 1.82$ was used as a measure of the size of the Q_A^--Fe^{2+}-signal. (D) Correlation between the loss of oxygen evolution and the reduction of Q_A (open symbols) or the formation of the 'split pheophytin' signal (filled symbols) as a function of photoinhibition of Photosystem-II-enriched membranes. The data are taken from several experiments in different sets of samples.

neously while pheophytin$^-$ does not give rise to the signal in the absence of Q_A^-. Therefore, the 'split pheophytin' signal is a measure of reducible Q_A rather than of reducible pheophytin [42] and the results in Fig. 3B–D further support the observation that the formation of Q_A^- was inhibited in correlation with the inhibition of the oxygen evolution.

The splitting of the signal, 30–35 G, is typical for the signal described in the presence of formate [39] while the splitting in the absence of formate is about 40 G

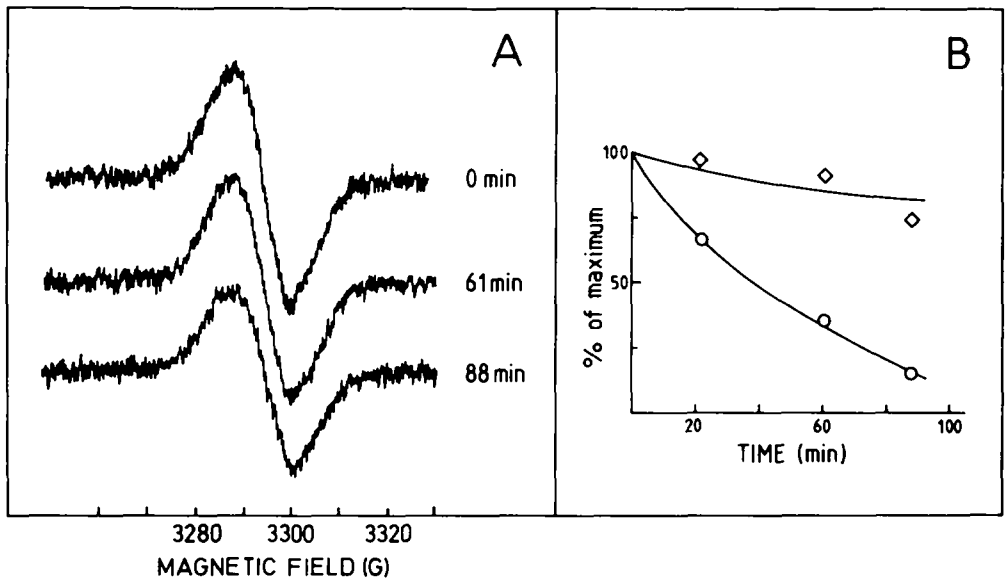


Fig. 4(A) Effect of photoinhibition for various times on the formation of the free-radical signal from pheophytin$^-$ induced by extensive illumination for 25 min at 20 °C of photoinhibited samples in the presence of dithionite. EPR conditions: temperature, 15 K; microwave power, 65 dB down from 200 mW; modulation amplitude, 2.5 G; microwave frequency, 9.239 GHz. (B) Time-course for the inhibition of the oxygen evolution (circles) and the formation of the free-radical pheophytin$^-$ signal at $g = 2.0036$ (diamonds) induced by photo-accumulation of pheophytin$^-$ in photoinhibited Photosystem-II-enriched membranes.

[38,39]. No contribution of the latter spectrum could be observed in the photoinhibition experiment, which suggests that loss of formate of formate binding early during the photoinhibition was not the cause for the reduced size of the Q_A^--Fe^{2+} signal.

The spectrum in Fig. 3B is a mixture of the 'split pheophytin' signal and a large radical signal in the center of the spectrum. The amplitude of the 'split pheophytin' signal saturates with the illumination at 198 K (Fig. 3B inset) and the spectra presented in Fig. 3B were recorded after saturating illumination. The middle part of the spectrum does not saturate similarly with illumination (Fig. 3B inset) and a signal with similar properties has been described earlier [42].

The total amount of photochemically reducible pheophytin can be measured by photo-accumulation of reduced pheophytin using extensive illumination under reducing conditions at room temperature [38,41]. Under these experimental conditions, Q_A first becomes doubly reduced and subsequently pheophytin is trapped in the reduced form (the electron donor to $P680^+$ is probably dithionite), which gives rise to a free-radical EPR signal distinguishable through its high g value ($g = 2.0037$) and large linewidth (13–14 G) [41]. Fig. 4 shows the results from such an experiment in photoinhibited Photosystem II enriched membranes. Samples photoinhibited for various times were reduced by dithionite and then illuminated at 20 °C for 25 min, which was saturating at this temperature (not shown). Fig. 4A shows the radical region of the EPR spectrum recorded after this treatment and the signal present has the correct g value ($g = 2.0036$) and linewidth (13 ± 1 G) to be assigned to reduced pheophytin. The size of the signal (measured after saturating illumination) in the control sample amounted to 1.0–1.1 radical per Photosystem II reaction center as compared to Signal II_{slow}. The amplitude of the pheophytin$^-$ radical decreased during the photoinhibitory treatment but the decrease was much slower than the inhibition of oxygen evolution (Fig. 4B). This suggests that the primary charge separation reaction is still operational even though the capacity to transfer electrons to Q_A has been lost.

Effects of photoinhibition on the redox-potential of cytochrome b-559

In the photoinhibition experiments we recorded the EPR spectrum of oxidized cytochrome *b*-559 around $g = 3$ (the g_z peak). Fig. 1B shows the g_z peak in the cytochrome EPR spectrum and in this region of the spectrum the different potential forms can be distinguished. The high potential form is characterized by $g_z = 3.06$ while the different low potential forms have g values between $g = 2.94$-2.98 [43]. In the dark control, approx. 30% of the oxidized cytochrome was in the high-potential form and 70% was in the low-potential form. The EPR spectra in Fig. 1B were integrated and it was found that approx. 0.6 cytochrome molecules per reaction center were present in the oxidized form in the control membranes before photoinhibition. Assuming two molecules of cytochrome *b*-559 per reaction center [44] this corresponds to 30% of the cytochrome present. This fraction of oxidized cytochrome *b*-559 is normal in intact thylakoids or Photosystem-II-enriched membranes [45]. During photoinhibition, cytochrome *b*-559

progressively became oxidized to a larger extent and after 90 min of illumination nearly 2 cytochromes were oxidized per Photosystem II reaction center (Fig. 1A,B). The kinetics for the progressive oxidation of the cytochrome correlated with the inhibition of the oxygen evolution both in thylakoid membranes (Fig. 1A) and in Photosystem-II-enriched membranes (not shown).

The spectra in Fig. 1B show that the cytochrome *b*-559 that became oxidized during photoinhibition mainly had a *g*-value of $g < 3$, i.e., the dominating part of the oxidized cytochrome was in the low-potential form [43]. This was confirmed by optical measurements which revealed that the oxidized cytochrome could be reduced by dithionite but not by hydroquinone (not shown). Thus, the inhibition of oxygen evolution by strong illumination is closely connected to a potential shift of the cytochrome from its high-potential to its low-potential form.

Discussion

In this report we have used EPR spectroscopy to study the effects of photoinhibition on electron transfer reactions in Photosystem II *in vitro* in thylakoid membranes and Photosystem-II-enriched membranes. The formation of the S_2 state multiline EPR signal was inhibited during photoinhibition with the same kinetics as the inhibition of the oxygen evolution (Fig. 2; Ref. 14). This suggests that Q_A rather than Q_B is the primary target for the photoinhibition. The reason is that the multiline signal was induced by illumination at 198 K. At this temperature only one stable charge separation can occur in Photosystem II, since the electron transfer between Q_A and Q_B is blocked [30] and, in fact, the electron never reaches Q_B. Thus, it is likely that the damage induced in Photosystem II by the strong illumination should be sought in the electron transport chain between the Mn cluster and the primary quinone acceptor, Q_A.

We have considered three possible reasons for the inhibition of multiline signal formation; (i) The Mn-complex has been destroyed but the primary photochemistry including charge separation and charge stabilisation is left intact; (ii) The electron transfer from pheophytin$^-$ to Q_A is inhibited; (iii) The primary charge separation reaction is inhibited.

It was possible to distinguish (i) from (ii) and (iii) from our results obtained after an illumination at 198 K of photoinhibited samples. It is known that, at 198 K, the kinetically preferred donor to $P680^+$ is the Mn cluster. If this is impaired, cytochrome *b*-559 or a chlorophyll molecule can act as alternative electron donors to $P680^+$ [43,46]. The donation is sequential, with the chlorophyll first reducing $P680^+$ and then cytochrome *b*-559 reducing the oxidized chlorophyll [47]. In intact thylakoids and Photosystem-II-enriched membranes illumination at 198 K results in the oxidation of some chlorophyll (partly this originates from $P700^+$ which has EPR parameters similar to those of the chlorophyll radical from Photosystem II). However, the induction of this signal did not increase as a consequence of photoinhibition. Furthermore, the results show that photoinhibition did not increase the amount of cytochrome *b*-559 that was oxidized by the secondary illumination, either in the thylakoids or in Photosystem-II-enriched membranes.

Taken together these results show that the total amount of oxidized electron donors upon low temperature illumination is decreased together with the photoinhibition of oxygen evolution. Normally, $P680^+$ is reduced from the donor side of Photosystem II only when the electron has been transferred from pheophytin$^-$ to Q_A. Otherwise the primary radical pair $P680^+$-pheophytin$^-$ rapidly recombines and no electron will be taken from the donor side by $P680^+$. Therefore, the observed decreased oxidation of electron donors suggests that an important consequence of photoinhibition is an impairment of the photochemical reduction of Q_A.

There are two likely possibilities for an impairment of the charge stabilization on the acceptor side of Photosystem II. Either the function of Q_A to stabilize the negative charge is lost (alternative ii) or the primary charge separation reaction is blocked (iii). These possibilities were tested by measurements of the EPR signal from the reduced primary acceptor in photoinhibited samples and the results show that the amount of chemically or photochemically reducible Q_A^- was lowered in correlation with the inhibition of the oxygen evolution. In addition, the amplitude of the 'split pheophytin' EPR signal was decreased in correlation with the inhibition of the oxygen evolution (Fig. 3B–D). This strengthens the conclusion that photoinhibition results from inhibition of the reduction of Q_A since the 'split pheophytin' signal is observable only in the presence of reduced Q_A [38,41,42].

The interesting possibility that the primary charge separation reaction was not inhibited to the same extent as the Q_A reduction was tested by a quantification of photoreducible pheophytin. The results (Fig. 4) show that photoinhibition did not lower the amount of photoreducible pheophytin with the same kinetics as the oxygen evolution was inhibited. Instead, the fraction of photoreducible pheophytin was lost approx. 4–5-times more slowly than the oxygen evolution. Thus the primary charge separation reaction is still operational in a large fraction of the centers, even when the oxygen evolution and the formation of the S_2-state multiline EPR signal (compare Fig. 2) are completely abolished.

The main conclusion from these measurements is that the strong photoinhibitory illumination impairs the function of Q_A while the primary charge separation reaction is left intact. This interpretation is in contradic-

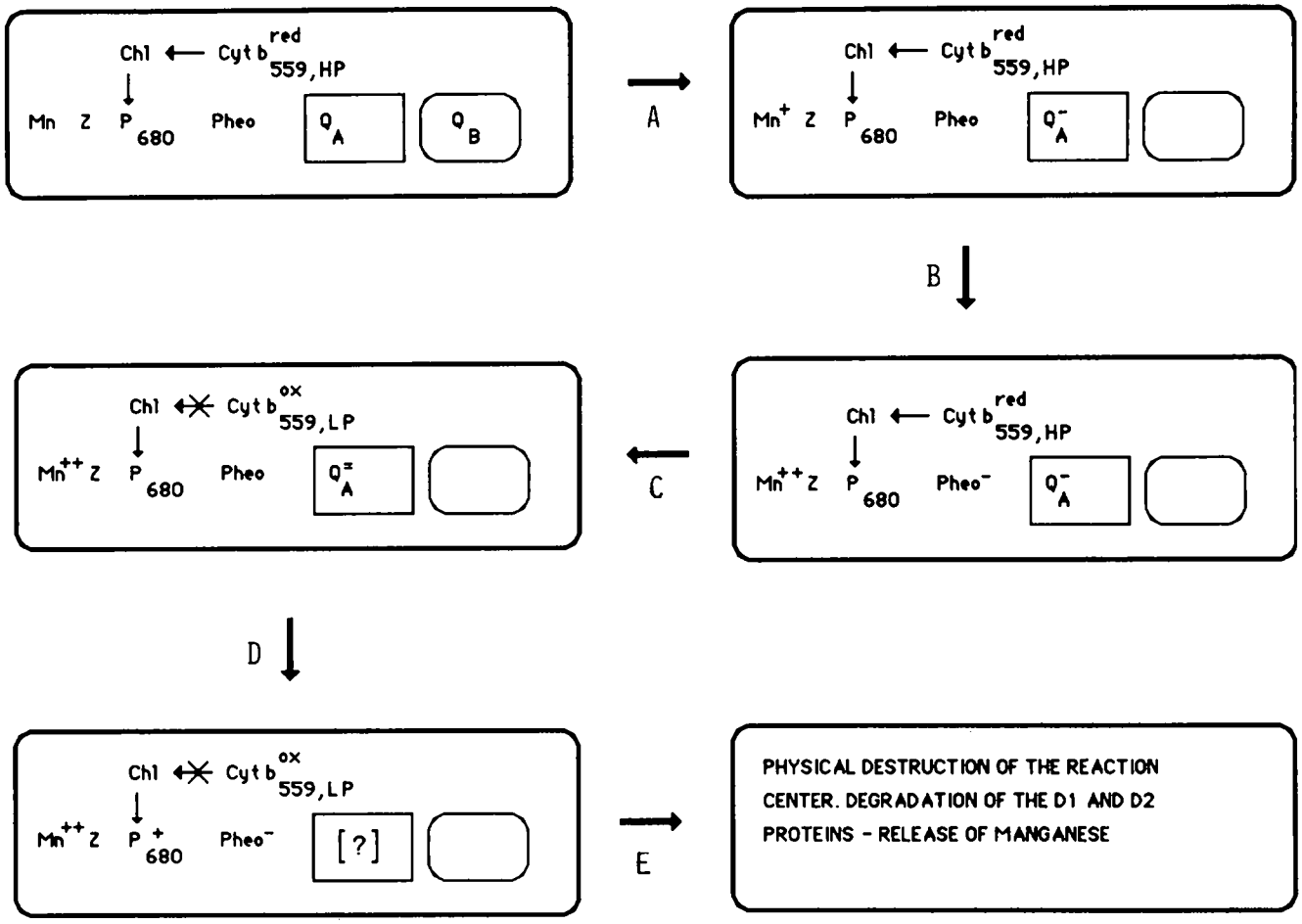
tion to the, until recently predominating, hypothesis that the origin for photoinhibition is the accumulation of Q_B^{2-} in the Q_B site [16,20]. Also at variance with the Q_B hypothesis, the simultaneous inhibition oif electron transport and both Q_A and Q_B reduction was observed recently in thermoluminescence experiments with isolated spinach thylakoid membranes [48]. Two models were offered to account for the data. Either electron transport was inhibited between pheophytin$^-$ and Q_A or Q_A and Q_B were damaged simultaneously. With thermoluminescence it was not possible to judge between these models, but in the light of our data the first hypothesis seems more likely.

Our results are in agreement with optical photo-accumulation experiments which described photoinhibition of electron transport without a loss of pheophytin reduction in a detergent-treated Photosystem II preparation [21]. However, other optical investigations, performed with similar experimental techniques, did not show photoreduction of pheophytin in photoinhibited samples, which led to the suggestion that the charge separation reaction was inhibited [22,23]. Two possible reasons for this discrepancy between the optical measurements (and our EPR study) can be pointed out. Firstly, it is likely that the length of the dark adaptation applied after photoinhibition before the measurement of photo-accumulated pheophytin might interfere with the measurements. The half-life-time for pheophytin$^-$ (when P680 is reduced) in the absence of Q_A is about 10 min (not shown; Rutherford, A.W. and van Mieghem, F., personal communication). Thus, if short dark adaptation is applied, no photoreducible pheophytin is observable since most of the pheophytin would have remained reduced from the start by the strong photoinhibitory illumination applied. The dark adaptation times used are not clear from the literature [21–23], but in the present EPR work it took approx. 30 min between the photoinhibition and the freezing of the EPR samples (centrifugation plus dark incubation). A second alternative is that the photosynthetic preparations, which differ between the studies, behave differently with respect to the mechanism for photoinhibition.

The model [16,17] that photoinhibition and the degradation of the D1 protein is caused by the accumulation of reduced species of Q_B in the Q_B site has leaned heavily on differences in the inhibition of electron transport between water and silicomolybdate (thought to accept electrons directly from Q_A) or quinone type electron acceptors (accepting electrons in the Q_B site). However, as discussed by Chritchley [2], these interpretations have been weakened by recent investigations on the function of silicomolybdate [49]. In addition, it is known that the D_1 protein carries many of the redox components in Photosystem II which makes it possible to reconcile degradation of the D_1 protein, not only with the function of Q_B, but also with the function of most other redox components and electron transfer reactions in Photosystem II [50].

The involvement of donor side reactions in photoinhibition have been invoked from experiments with inhibited water oxidation [18,24]. These results clearly do not represent the same inhibition type as we study here. When the water splitting is inhibited, the acceptor side reactions in Photosystem II are much more efficient than the electron donation to $P680^+$. Therefore, illumination results in the formation of highly oxidizing species on the oxidative side of Photosystem II which are likely to induce irreversible damage. However, under our conditions the water oxidation is intact, rapidly reducing Tyr_Z^+ and $P680^+$, leaving no harmful 'electron holes' on the oxidizing side. Instead, it seems that the acceptor side of Photosystem II cannot cope with the rapid electron flow through Photosystem II at high intensities. Ultimately this leads to the inhibition of electron transfer to Q_A.

What is the fate of Q_A following photoinhibition? Our results and interpretations are summarized in Scheme I. One likely explanation for our results is that the intense illumination leads to double reduction of Q_A in a low quantum-yield reaction. Normally, Q_A accepts only one electron which then is transferred to the two-electron acceptor Q_B. Under strong continuous illumination in the absence of exogenous electron acceptors the plastoquinone pool is reduced faster than it is reoxidized. Under these circumstances Q_A^- will be the dominating species in the acceptor side complex of Photosystem II, while the Q_B site is likely to be unoccupied (Scheme I, A). The illumination continuously induces charge separation reactions in the reaction center leading to formation of the primary radical pair which in the presence of Q_A^- recombines in a few nanoseconds [3,4]. The recombination reaction takes place in a majority of the centers. However, the electron donation from Tyr_Z to $P680^+$ is very fast (30–250 ns) [51] and it is likely that $P680^+$ is reduced by electron donation from Tyr_Z (and ultimately from water) with a low quantum yield. In those centers the electron is trapped on pheophytin (Scheme I, B). Pheophytin$^-$ is a fairly strong reductant ($E_m \approx -610$ mV [52]) and it is likely that it can reduce Q_A^- a second time (Scheme I, C). This reaction is known to occur in reaction centers from photosynthetic bacteria when they are illuminated under reducing conditions. Similar reactions have also been proposed to occur in Photosystem II when it is illuminated at room temperature in the presence of dithionite or even in the dark at redox potentials lower than approx. -350 mV [38,41,53]. The fate of Q_A^{2-} is unknown, but it is likely that it leaves the Q_A site (Scheme I, D). From in vitro experiments performed under strongly reducing conditions it was proposed that the double reduction of Q_A and the subsequent formation of the quinol leads to a loss of QH_2 from the Q_A



Scheme I.

site [38,53]. In Photosystem-II-enriched membranes, partial reversibility of this reaction was reported [38] but in a recent study such reversibility was not observed [53]. In vivo it is quite likely that Q_A^{2-}, while still in the Q_A site, can be partially reoxidized by plastoquinone if the strong illumination ceases. If this is the case the presumed loss of QH_2 from the Q_A site would constitute the first irreversible step during the photoinhibition (Scheme I, D).

We have shown that the inhibition of the electron transport is followed by a slow degradation of the reaction center proteins (Fig. 1A, 14). It is likely then, that the light reactions giving rise to the damage to the proteins, is in fact the primary charge separation reaction or donor side reactions in perturbed centers from which Q_A has been lost (Scheme I, D and E). This is in accordance with the results from studies using Photosystem II preparations inhibited at the level of water oxidation, which showed that in this case donor side reactions were responsible for the inhibition of electron transport [18,24] and also possibly for the degradation of the D_1 and D_2 proteins [18]. In this respect, the recent proposal that one function of cytochrome *b*-559 is to protect the reaction center from damaging photooxidation reactions caused by the action of $P680^+$ when the electron donor system is incompetent is very interesting [47]. The data presented here show that cytochrome *b*-559 is oxidized early during photoinhibition before the degradation of the reaction center proteins (Scheme I, C). It can therefore not exert its suggested protective function when it is best needed, although $P680^+$ is formed continuously. The continued illumination in the absence of the protective cytochrome *b*-559 would then lead to destruction of the reaction center and ultimately to degradation of the reaction-center proteins D_1 and D_2 in low quantum-yield reactions (Scheme I, E). The protein chemical reactions involved in step E in Scheme I are not understood, but they are likely to involve both photochemical steps and proteinase-dependent reactions.

It was earlier shown that the inhibition of electron transport preceeds the major changes among the Photosystem II proteins [14]. However, we observe an early shift of the potential of cytochrome *b*-559 to the low potential form. The potential shift of cytochrome *b*-559 is due to a subtle conformational change of the histidine

ligand to the iron [54] which shows that also the early reactions during the inhibition of electron transport results in conformational changes in reaction-center proteins. A more elaborate study on the effects of photoinhibition on different proteins and the structural organization of Photosystem II will be presented elsewhere.

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