Characterization of Chlorophyll Triplet Promoting States in Photosystem II Sequentially Induced during Photoinhibition†

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ABSTRACT: It has recently been demonstrated that strong illumination under anaerobic conditions leads to the double reduction of the primary quinone acceptor, Q_A, which in turn promotes the light-induced formation of triplet reaction center chlorophyll, $^3P_{680}$, a potentially dangerous species to its protein surroundings in the presence of oxygen [Vass, I., Styring, S., Hundal, T., Koivuniemi, A., Aro, E.-M., & Andersson, B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1408–1412]. Here we have studied in further detail the formation of $^3P_{680}$ producing centers in anaerobically photoinhibited photosystem II membranes by using low-temperature EPR spectroscopy. The results show that $^3P_{680}$ formation occurs in three different populations of modified photosystem II centers. After a short period of photoinhibitory illumination, a very stable form of singly reduced Q_A is observed, with a decay halftime of several minutes at room temperature, and our results indicate that already this population of centers promotes the light-induced formation of the spin-polarized EPR signal from $^3P_{680}$. The formation of these centers is enhanced below pH 6.0, indicating the involvement of a protonation event in neutralizing the negative charge on Q_A^-, a prerequisite for efficient primary charge separation and subsequent triplet formation via the radical pair mechanism. If these centers are incubated in the dark, the stable singly reduced Q_A species is slowly reoxidized concomitant with the loss of its triplet forming ability. Extended photoinhibitory illumination converts the stable form of singly reduced Q_A to an EPR-silent species indicating the second reduction of Q_A^-. The second negative charge on the double-reduced Q_A is neutralized most likely by a second protonation. This state also promotes $^3P_{680}$ formation. In the final stage of anaerobic photoinhibition the double-reduced and protonated Q_A is suggested to leave or be displaced from its binding site to form the third population of triplet P_{680} promoting photosystem II centers. The strong anaerobic illumination also leads to an alteration at the donor side via reduction of tyrosine-D^+.

The reaction center in photosystem II (PSII)[1] is composed of the D1 and D2 proteins that form a heterodimer (Nanba & Satoh, 1987) in close structural and functional homology to the reaction center complex of purple bacteria [for recent reviews, see Hansson and Wydrzynski (1990), Andersson and Styring (1991), and Debus (1992)]. The D1/D2 heterodimer harbors the redox cofactors required for the primary steps of light-induced electron transport: the primary electron donor chlorophyll(s), P_{680}; the first pheopytin electron acceptor, Phe; the primary, Q_A, and secondary, Q_B, quinone electron acceptors; and the redox active tyrosine residues, Tyr_Z on the D1 protein and Tyr_D on the D2 protein. The catalytic manganese cluster involved in the water-oxidation reaction is probably also bound to the D1/D2 heterodimer [see Andersson and Styring (1991) and references therein].

Exposure of oxygenic photosynthetic organisms to high light intensities inhibits photosynthetic electron transport. This phenomenon, denoted photoinhibition, is targeted to the reaction center of PSII and results in the subsequent degradation of the D1 reaction center protein [see reviews by Powles (1984), Andersson and Styring, (1991), Barber and Andersson (1992), and Prasil et al. (1992)]. Recent studies have demonstrated that strong illumination can impair electron transport both at donor and at acceptor sides of PSII. Donor side photoinhibition most likely affects the functioning of Tyr-Z or P_{680} (Callahan et al., 1986; Jegerschöld et al., 1990; Blubaugh et al., 1991; Eckert et al., 1991). Considering the acceptor side induced photoinhibition, the primary lesion in the electron transport was earlier thought to occur at the secondary quinone acceptor Q_B (Kyle et al., 1984; Ohad et al., 1988; Kirilovsky et al., 1988) or at the primary charge separation step (Cleland et al., 1986). However, recent spectroscopic studies have provided strong evidence that the function of Q_A is impaired due to double reduction during the early phase of photoinhibition (Setlik et al., 1990; Styring et al., 1990; Vass et al., 1992). Double-reduced Q_A is an abnormal state that promotes the formation of $^3P_{680}$ with a high yield during illumination (van Mieghem et al., 1989, 1992; Vass et al., 1992). $^3P_{680}$ in turn, interacts with molecular oxygen leading to the production of singlet oxygen. Singlet oxygen is highly reactive (Asada & Takahashi, 1987; Cogdell & Frank, 1987), and its damaging effects are a very likely reason for the subsequent triggering and degradation of the D1 protein (Vass et al., 1992).

High yield of $^3P_{680}$ formation in PSII has earlier been shown to be facilitated by chemical double reduction (and presumed

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[1] Abbreviations: Chl, chlorophyll; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; P_{680}, primary electron donor of PSII; $^3P_{680}$, triplet state of P_{680}; PSI, photosystem I; PSII, photosystem II; Phe, pheophytin; PpBQ, phenyl-*p*-benzoquinone; Q_A, primary quinone acceptor of PSII; Q_B, secondary quinone acceptor of PSII; Tyr_Z, tyrosine-161 on the D1 protein (electron carrier between P_{680} and the water-oxidizing complex); Tyr_D, tyrosine-161 on the D2 protein (accessory electron donor in PSII).

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protonation) of Q_A (van Mieghem et al., 1989) or by its absence from the binding site (Okamura et al., 1987; Durrant et al., 1990). It was recently shown that both double reduction and disconnection of Q_A from its binding site may occur as a consequence of strong illumination under anaerobic conditions leading to the buildup of $^3P_{680}$ promoting PSII centers (Vass et al., 1992). However, we also observed high yield of $^3P_{680}$ formation in the earlier phases of anaerobic photoinhibition in centers which we proposed to contain singly reduced Q_A (Vass et al., 1992). This is highly surprising and seemingly contradicts the generally accepted idea that the presence of Q_A^- in a PSII reaction center actually suppresses the primary charge separation reaction, and consequently $^3P_{680}$ formation, due to the repulsive interaction between Q_A^- and Phe^- (Schatz & Holzwarth, 1986; Schatz et al., 1987, 1988; van Mieghem et al., 1989, 1992; Vass & Styring, 1992). An attractive resolution of this apparent contradiction would be the neutralization of the negative charge on Q_A^-, and we have recently provided fluorescence measurements and theoretical calculations (Vass et al., 1992; Vass & Styring, 1992) that support this hypothesis.

Here we have characterized in further detail the triplet producing PSII centers which are formed during strong anaerobic illumination. The results indicate that $^3P_{680}$ formation may occur in the presence of singly reduced Q_A. Experimental results are presented indicating that a protonation event leads to the neutralization of the negative charge on Q_A^-. A reaction sequence leading to the stepwise double reduction and double protonation of Q_A is also proposed.

MATERIALS AND METHODS

Sample Preparation. Spinach thylakoids were isolated by standard procedures and stored in 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, 20 mM Hepes, pH 7.5, at 3–4 mg of Chl/mL at −80 °C until use. PSII-enriched membranes from spinach thylakoids were prepared according to Berthold et al. (1981) with the modifications described by Ono and Inoue (1985) and stored at −80 °C in 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, 20 mM Mes, pH 6.3, at 3–4 mg of Chl/mL.

For anaerobic photoinhibition, PSII membranes at a concentration of 1.5–2.5 mg of Chl mL^{-1} were gently flushed in EPR tubes for 5 min with argon to remove oxygen. The tubes were sealed and subsequently illuminated at room temperature with white light through a 5-cm-thick copper sulfate heat absorbing solution at 3000 $\mu E\ m^{-2}\ s^{-1}$ intensity, for different periods of time. After illumination, the samples in the EPR tubes were quickly frozen (in about 1–2 s if not otherwise indicated) and kept in liquid nitrogen until the EPR measurements which were performed within a few hours after photoinhibition.

To study the pH-dependent formation of $^3P_{680}$ promoting centers, PSII membranes were washed with a medium of 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, 2 mM Mes, pH 6.3, and then suspended at different pHs in the above medium but with 40 mM L-glutamic acid (pH 4.5–5.0), 40 mM Mes (pH 5.0–7.0), 40 mM Hepes (pH 7.0–8.0), or 40 mM glycyl-glycine (pH 8.0–8.5) as buffering agents.

In experiments where the $Q_A^-Fe^{2+}$ signal was measured, the samples were incubated with 50 mM formate for 10 min prior to the photoinhibitory illumination treatment to enhance the size of the signal (Vermaas & Rutherford, 1984). The full size of the $Q_A^-Fe^{2+}$ signal was measured after incubating the samples in the presence of 40 mM dithionite for 10 min in the dark at room temperature. Chemical double reduction of Q_A was achieved by incubating PSII membranes with 50 mM dithionite and 30 μM benzyl viologen in the dark at room temperature for 5 h as described by van Mieghem et al. (1989).

Oxygen Evolution Measurements. Steady-state rates of oxygen evolution were measured in a Hansatech oxygraph at saturating light with 1 mM phenyl-*p*-benzoquinone (P*p*BQ) as electron acceptor.

EPR Measurements. X-band low-temperature EPR spectra were recorded at 9.234 GHz with a Bruker ESP 300 spectrometer equipped with an Oxford Instruments helium cryostat and temperature controller. Data acquisition and data handling were performed with the ESP 300 program of the EPR spectrometer. For light-induced generation at 4 K of the spin-polarized $^3P_{680}$ EPR signal, the samples were illuminated in the EPR cavity with a 1000-W tungsten projector through a 5-cm-thick copper sulfate heat absorbing solution. The triplet spectra were obtained as the light minus dark difference spectra.

RESULTS

Buildup of $^3P_{680}$ Forming Centers during Anaerobic Illumination. The inactivation of electron transport, as a consequence of anaerobic photoinhibition, was followed by parallel measurements of EPR signals from different redox components in PSII.

The functional intactness of the primary quinone acceptor, Q_A, can be directly monitored by EPR spectroscopy since the magnetic interaction between the singly reduced quinone, Q_A^-, and the nearby acceptor side iron, Fe^{2+}, gives rise to a well-characterized EPR signal [for a recent review, see Miller and Brudvig (1991)]. In contrast, the oxidized or double-reduced states of Q_A are diamagnetic and thus, EPR silent. In the nonphotoinhibited PSII membrane particles the $Q_A^-Fe^{2+}$ EPR signal is absent in the dark (Figure 1A, solid curve a). However, the $Q_A^-Fe^{2+}$ EPR signal can be quantitatively induced in these centers by reducing Q_A in the dark with dithionite (Figure 1A, dotted curve a).

Five minutes of room-temperature illumination at anaerobic conditions of the PSII membranes induced a large $Q_A^-Fe^{2+}$ EPR signal (Figure 1A, curve b), with an amplitude corresponding to about 70% of the maximum dithionite-induced signal obtained in the nonphotoinhibited control (Figure 1A, dotted curve a). The $Q_A^-Fe^{2+}$ signal, which was produced by the anaerobic illumination, was very stable in the dark at room temperature and decayed with a half-time of several minutes (see below). Further illumination gradually decreased the $Q_A^-Fe^{2+}$ signal, and after 20 min of illumination about 20% of the signal remained (Figure 1A, curve c).

The light-induced decrease of the $Q_A^-Fe^{2+}$ EPR signal indicates that Q_A is either double reduced or lost (disconnected) from its binding site. Both of these conditions are known to facilitate the light-induced formation of $^3P_{680}$ (Okamura et al., 1987; van Mieghem et al., 1989). If inducible in a sample, $^3P_{680}$ gives rise to a highly specific spin-polarized chlorophyll triplet EPR signal that can be observed by EPR spectroscopy during illumination at 4 K. Here we have measured the ability of anaerobically photoinhibited PSII samples to promote $^3P_{680}$ formation. In agreement with previous results, no $^3P_{680}$ formation was observed in the nonphotoinhibited control samples (Figure 1B, solid curve a). The triplet signal could be observed after chemical double reduction (Figure 1B, dotted curve a) or after anaerobic photoinhibition of the samples (Figure 1B, curves b and c). The fraction of $^3P_{680}$ forming PSII centers gradually increased during the course of the photoinhibitory treatment (Figures 1B and 2B). This supports earlier suggestions that photoinhibition may lead to double

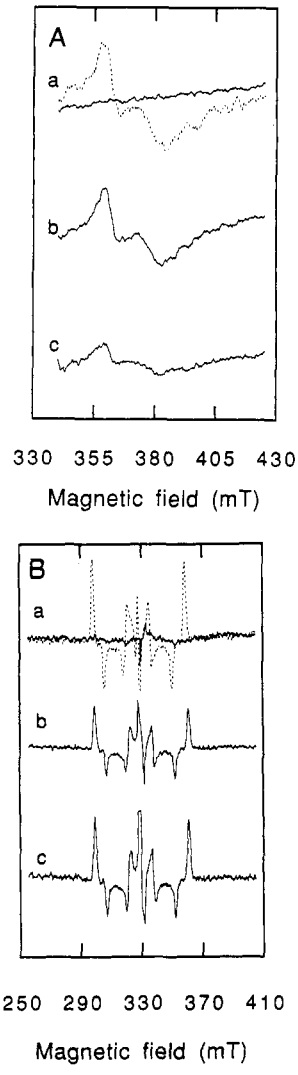


FIGURE 1: Effect of strong anaerobic illumination on the $Q_A^-Fe^{2+}$ and $^3P_{680}$ EPR signals. PSII membranes were anaerobically illuminated in EPR tubes. The illuminated samples were frozen within 1–2 s after the strong illumination was stopped. (A) The $Q_A^-Fe^{2+}$ EPR signal was measured either without further treatment (solid spectra) or after 10 min of dark incubation in the presence of 40 mM dithionite at room temperature (dotted spectrum) on samples which had been photoinhibited for (a) 0 min, (b) 5 min, or (c) 20 min. EPR conditions: temperature, 4 K; microwave power, 32 mW; modulation amplitude, 3.2 mT. (B) The $^3P_{680}$ EPR signal was measured during illumination either without further treatment after photoinhibition (solid spectra) or after quantitative double reduction of Q_A as described in Materials and Methods (dotted spectrum). The triplet measurements were performed in the same samples as that of the $Q_A^-Fe^{2+}$ signal. EPR conditions: temperature, 4 K; microwave power, 63 μW; modulation amplitude, 2.2 mT.

reduction and subsequently to disconnection of Q_A from its site (van Mieghem et al., 1989; Styring et al., 1990; Vass et al., 1992).

However, the results presented in Figure 1B indicate that $^3P_{680}$ formation not only occurs in PSII reaction centers with double-reduced Q_A but also occurs to a large extent in reaction centers containing Q_A^-. The amplitude of the $Q_A^-Fe^{2+}$ signal recorded in a sample after 5 min of anaerobic illumination suggests that it represents about 70% of the PSII centers (Figure 1A, spectrum b). In the same sample the light-induced $^3P_{680}$ signal (Figure 1B, spectrum b) was about 55% of the maximal signal size observed after chemical double reduction of Q_A in a nonphotoinhibited control sample (Figure 1B, dotted spectrum a). Thus, it is reasonable to suggest, in accordance with our earlier hypothesis (Vass et al., 1992), that short

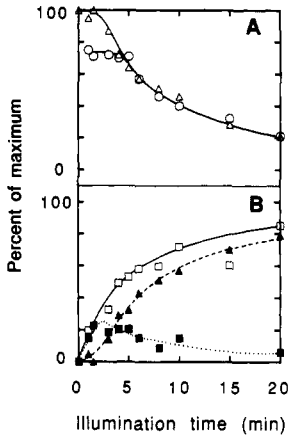


FIGURE 2: Time course of changes in the $Q_A^-Fe^{2+}$ and $^3P_{680}$ EPR signals during strong anaerobic illumination. The $Q_A^-Fe^{2+}$ and $^3P_{680}$ EPR signals were measured in the same samples which had been exposed to anaerobic illumination, as in Figure 1. (A) Amplitude of the $Q_A^-Fe^{2+}$ signal as a function of the illumination time. The 100% value in the 0-min sample represents the size of the $Q_A^-Fe^{2+}$ signal which was induced by dithionite reduction in the dark in the nonphotoinhibited control. In the photoinhibited samples the amplitude of the $Q_A^-Fe^{2+}$ signal is shown both immediately after the illumination (○) and following dithionite reduction (△). (B) Amplitude of the $^3P_{680}$ EPR signal as a function of illumination time (□). The 100% value represents the amplitude of the triplet signal that was induced by chemical double reduction of Q_A in the nonphotoinhibited control. The fraction of PSII centers that have lost the capacity to form the $Q_A^-Fe^{2+}$ EPR signal (▲) was obtained from the data in Figure 2A. The fraction of PSII centers that promote $^3P_{680}$ formation in the presence of the $Q_A^-Fe^{2+}$ EPR signal (■) was obtained by subtracting the fraction of centers that have lost the capacity to form the $Q_A^-Fe^{2+}$ EPR signal (▲) from the total amount of triplet forming centers (□).

anaerobic photoinhibition induces a substantial fraction of PSII centers in which a singly reduced Q_A species promotes the formation of $^3P_{680}$.

The formation of $^3P_{680}$ in the presence of what seems to be singly reduced Q_A is surprising and seemingly contradictory to the idea of electrostatic control of primary charge separation reaction (Schatz et al., 1987, 1988; van Mieghem et al., 1989, 1992; Vass & Styring, 1992). We therefore performed experiments to test whether the $^3P_{680}$ and the $Q_A^-Fe^{2+}$ EPR signals may indeed arise from the same PSII center. The time course for the induction of these signals during strong anaerobic illumination was carefully measured (Figure 2A,B). Shortly after the onset of the strong illumination, Q_A was reduced and the amplitude of the $Q_A^-Fe^{2+}$ EPR signal represented approximately 75% of the PSII centers after a 30-s illumination (Figure 2A). The amplitude of the $Q_A^-Fe^{2+}$ EPR signal measured immediately after the photoinhibitory illumination remained constantly high for about 5 min. Thereafter the signal size decreased, and after 20 min of photoinhibition it represented about 20% of the PSII centers. In each sample, including the nonphotoinhibited control, the amplitude of the total inducible $Q_A^-Fe^{2+}$ signal was measured after reduction of the still oxidized Q_A fraction with dithionite. The chemical reduction resulted in an increase of the $Q_A^-Fe^{2+}$ EPR signal in the early phase of photoinhibition (<4 min) (Figure 2A). This shows that at this stage of the photoinhibitory process Q_A^- could be quickly reoxidized in part of the PSII centers. In samples that had been photoinhibited for longer times (>5 min) the chemical reduction did not increase the $Q_A^-Fe^{2+}$ signal, which shows that Q_A was either in a stable singly reduced or in an EPR-silent doubly reduced form.

The decrease of the $Q_A^-Fe^{2+}$ EPR signal during photoinhibition is not exponential and the experiments reveal, very

reproducibly, a lag phase in the decline of the $Q_A^-Fe^{2+}$ signal during the first few minutes of illumination, measured either immediately after the illumination or after the addition of dithionite (Figure 2A). The exact duration of the lag phase varied with sample concentration and light intensity. It is also important to note that the spectral shape of the $Q_A^-Fe^{2+}$ signal was the same throughout the entire illumination period (Figure 1A, curves a–c).

The $^3P_{680}$ EPR signal, which was measured in the same samples before the addition of dithionite, increased progressively during the 20-min illumination to about 80% of the maximal amplitude (Figure 2B) that can be observed after chemical double reduction of the nonphotoinhibited control samples (compare also Figure 1B spectrum b and the dotted spectrum a).

The important question is then, which centers give rise to the $^3P_{680}$ signal, centers with singly reduced or with doubly reduced Q_A? The observed decrease in the total amplitude of the $Q_A^-Fe^{2+}$ EPR signal, measured in the presence of dithionite, indicates that Q_A becomes either double reduced or disconnected from its binding site after prolonged photoinhibition. If $^3P_{680}$ could be induced only in centers with double-reduced or lost Q_A, the decrease of the total $Q_A^-Fe^{2+}$ signal (triangles in Figure 2B) and the increase in the formation of $^3P_{680}$ (squares in Figure B) should proceed in parallel during the course of the anaerobic photoinhibition. This would be similar to the situation which occurs during chemical double reduction of Q_A (van Mieghem et al., 1989). However, during anaerobic photoinhibition this is not the case! Instead, the triplet forming centers are built up faster than the PSII centers with singly reduced Q_A are lost (Figure 2B). The difference between the two curves (Figure 2B, dotted curve) represents centers in which $^3P_{680}$ is formed in the presence of singly reduced Q_A. This population comprises the dominating fraction of $^3P_{680}$ forming centers in the very early phase of photoinhibition and reaches a maximum at about 25% of the centers after approximately 3–4 min of illumination (Figure 2B, dotted curve). Thus, these results strongly suggest that a population of PSII centers that promote $^3P_{680}$ formation in the presence of Q_A^- is formed in the early phase of anaerobic photoinhibition.

During the continued illumination, the Q_A^- containing and $^3P_{680}$ promoting centers are converted to other populations of PSII centers which also promote $^3P_{680}$ formation but now in the absence of Q_A^-. These centers dominate after 10–15 min of illumination. The exact time courses for the buildup of these different PSII populations were dependent on the sample concentration and the light intensity, but the same general behavior was always seen.

A similar behavior with rapid buildup of triplet forming centers was also observed in anaerobically photoinhibited thylakoid membranes (not shown) which provide a more intact photosynthetic material than the PSII membranes.

pH-Dependent Buildup of $^3P_{680}$ Promoting PSII Centers during Photoinhibition. The normally low yield of $^3P_{680}$ formation in the presence of singly reduced Q_A is thought to relfect the suppression of the primary radical pair formation in PSII due to electrostatic repulsion between Phe^- and Q_A^- (Schatz et al., 1987, 1988; van Mieghem et al., 1989; Vass & Styring, 1992). The high triplet yield observed after double reduction of Q_A or in purified reaction center complexes which lack Q_A was explained by charge neutralization by protonation or by the absence of Q_A, both of which would remove the electrostatic effect (van Mieghem et al., 1989, 1992).

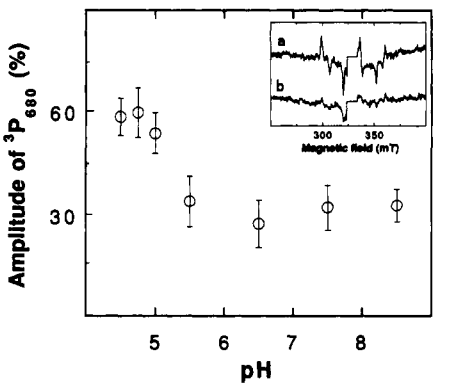


FIGURE 3: pH-dependent buildup of $^3P_{680}$ promoting PSII centers during strong anaerobic illumination. PSII membranes were suspended at different pHs and illuminated for 3 min. The relative amplitude of the light-induced $^3P_{680}$ EPR signal is plotted as a function of pH. The data represent the average of four experiments with the indicated standard deviation. The insert shows the $^3P_{680}$ signal at pH 6.5 before (a) and after incubation with 2 mM P*p*BQ for 5 min at room temperature (b).

The observation of high triplet yield in photoinhibited PSII centers which exhibit the $Q_A^-Fe^{2+}$ EPR signal (Figure 2B) suggests that the charge on Q_A^- is neutralized in these centers. A likely mechanism that could result in charge neutralization is protonation of Q_A^- or an amino acid residue in its close vicinity. In an attempt to test this hypothesis, the buildup of $^3P_{680}$ forming PSII centers was measured in PSII membranes which were anaerobically illuminated at different pHs for a constant period of time. On the basis of the data in Figure 2B, the duration of the strong illumination was chosen to 3 min to produce the maximal amount of triplet forming centers in which Q_A is singly reduced, with minimal interference from centers in which $^3P_{680}$ is formed in the absence of Q_A^-. The size of the $^3P_{680}$ EPR signal was practically constant above pH 6.0. However, the yield increased steeply between pH 6.0 and 4.5 (Figure 3) supporting the hypothesis that protonation events are involved in the buildup of $^3P_{680}$ promoting centers.

Incubation with P*p*BQ after photoinhibition has previously been shown to reoxidize the stable Q_A^- population and to diminish the related part of $^3P_{680}$ formation while $^3P_{680}$ formation in centers with doubly reduced Q_A is unaffected (Vass et al., 1992). Here the $^3P_{680}$ formed during the pH experiment was largely suppressible by P*p*BQ (Figure 3, inset). This further strengthens the conclusion that early during photoinhibition $^3P_{680}$ originates from a population of centers with reoxidizable Q_A species, most likely $Q_A^-(H^+)$.

Unfortunately, attempts to measure the $Q_A^-Fe^{2+}$ EPR signal under the same conditions led to inconclusive results. This is due to an earlier described pH-dependent shift of the *g* value for the $Q_A^-Fe^{2+}$ EPR signal from $g = 1.9$ at high pH to $g = 1.8$ at lower pHs (Rutherford & Zimmermann, 1984; Petrouleas & Diner, 1987), which is most likely the consequence of bicarbonate release at low pH. However, the results show that at low pHs, where the $^3P_{680}$ signal is almost quantitative, also a large $Q_A^-Fe^{2+}$ signal ($g = 1.8$ form) can be detected (data not shown).

Decay of Triplet Forming PSII Centers at Room Temperature. The results described above demonstrate the existence of a heterogeneous population of PSII centers, with different degrees of Q_A reduction, which promote $^3P_{680}$ formation in anaerobically photoinhibited PSII centers. In an earlier study the heterogeneity was revealed by the different stability of the various states as measured by the room temperature decay of their increased F_0 fluorescence (Vass et al., 1992).

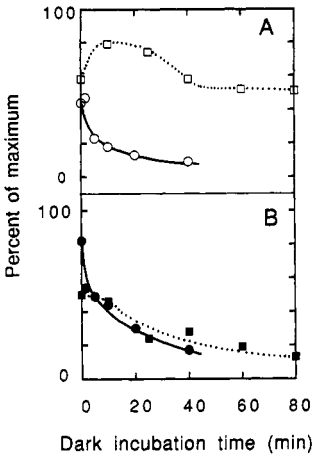


FIGURE 4: Room-temperature stability of the $^3P_{680}$ promoting centers and the $Q_A^-Fe^{2+}$ EPR signal during dark incubation after strong anaerobic illumination. PSII membranes were photoinhibited as in Figure 1 and the decrease of the light-inducible $^3P_{680}$ (○, □) (A) and of the dark-stable $Q_A^-Fe^{2+}$ EPR signals (●, ■) (B) was monitored during room temperature dark storage of samples which were photoinhibited for 3 min (○, ●) and 12 min (□, ■), respectively.

Here we have used EPR spectroscopy to directly detect the decay of the different triplet promoting states during room-temperature incubation in the dark. After 3 min of anaerobic illumination, the amplitude of the light-inducible triplet signal was 40–45% of the $^3P_{680}$ amplitude reached after 20 min of illumination, or 30–35% of the maximal triplet size. During dark storage, subsequent to the photoinhibitory illumination, the ability of $^3P_{680}$ formation showed a biphasic decay, with half-decay times of about 2–3 and 30 min (Figure 4A). About 70–80% of these $^3P_{680}$ promoting centers, corresponding to about 25% of the total PSII population, decayed with the short half-time. This fraction is approximately equal to the $^3P_{680}$ promoting centers that contain singly reduced Q_A (Figure 2B, dotted).

In a sample that was illuminated for 12 min, approximately 60% of the centers promoted $^3P_{680}$ formation immediately after the photoinhibition. During the first 10 min of the dark incubation, the light-inducible $^3P_{680}$ signal transiently increased to about 80% of the maximum. This transient increase was followed by a slow decay ($t_{1/2} \approx 30$ min) to a constant level at approximately 50% of the maximal triplet amplitude. After extended periods of anaerobic photoinhibition (>30 min), when the $Q_A^-Fe^{2+}$ signal had almost totally disappeared, we observed high yield of $^3P_{680}$ formation which remained completely unchanged during subsequent dark incubation (not shown).

In the same series of experiments, where the stability of the triplet promoting centers was monitored in the photoinhibited samples, the stability of the $Q_A^-Fe^{2+}$ EPR signal was also measured. After 3 min of anaerobic illumination the $Q_A^-Fe^{2+}$ EPR signal reached about 80% of the maximal amplitude, measured in the nonphotoinhibited control after dithionite reduction. During the first minute of the dark adaptation period about half of the $Q_A^-Fe^{2+}$ signal was lost (Figure 4B). The rest decayed slowly ($t_{1/2} \approx 30$ min). In the sample that was exposed to the anaerobic illumination for 12 min, the $Q_A^-Fe^{2+}$ EPR signal, which amounted only to 50% of the maximum amplitude, decayed monophasically ($t_{1/2} \approx 35$ min) after an apparent lag period of about 10 min (Figure 4B, dotted curve).

These results clearly demonstrate that, in agreement with our previous findings (Vass et al., 1992), there exist three different populations of $^3P_{680}$ promoting PSII centers induced during the anaerobic photoinhibition. Early during the strong illumination a large fraction of quite unstable (half-time for the decay ≈ 2–3 min) triplet forming states was induced. During further illumination this population is progressively transferred into more stable $^3P_{680}$ promoting states (half-time for the decay is 30–40 min) and later to a nondecaying population. The room-temperature decay of triplet forming PSII centers also explains the apparent loss of the $^3P_{680}$ signal in our previous study, when samples were dark adapted for 15 min after extended photoinhibitory illumination [Figure 3 in Vass et al. (1992)].

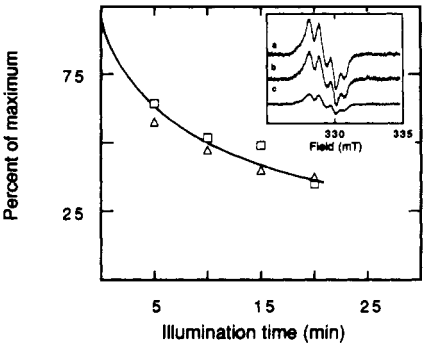


FIGURE 5: Time course for the changes of signal II_{slow} and oxygen-evolving activity during anaerobic photoinhibition. PSII membranes were anaerobically photoinhibited for different periods of time. The amplitude of signal II_{slow} (△) and the rate of oxygen evolution (□) is plotted as a function of the duration of the photoinhibitory illumination. One-hundred percent represents the value that can be observed in the nonphotoinhibited control. The insert shows the shape of signal II_{slow} after 0 min (a), 5 min (b), and 15 min (c) of anaerobic photoinhibition.

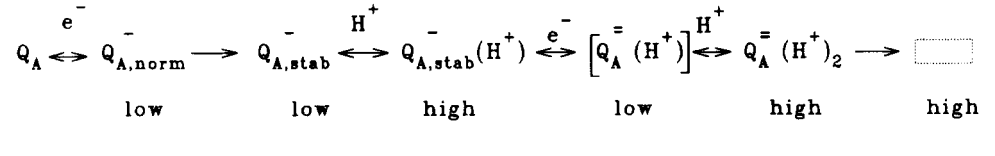
Changes at the Donor Side in PSII during Anaerobic Photoinhibition. The earlier studied triplet forming states of PSII, in which Q_A is chemically double reduced or absent (Rutherford & Mullet, 1981; Rutherford et al., 1981; Okamura et al., 1987; van Mieghem et al., 1989), are inactive at their donor side: The very long chemical treatment with dithionite and benzyl viologen (van Mieghem et al., 1989), which is used to double reduce Q_A, reduces Tyr_D^+ and probably also removes the Mn cluster. Similarly, in the isolated D1/D2 reaction center complexes the Mn cluster is removed during the isolation procedure, while Tyr_D is converted into its reduced form.

During anaerobic photoinhibition the EPR signal II_{slow}, arising from Tyr_D^+, was gradually decreased closely following the inhibition of oxygen evolution (Figure 5). However, the shape and the g value of the remaining signal II_{slow} did not change (Figure 5, inset). This situation is different from that observed after aerobic photoinhibition where the induction of a narrow radical at around $g = 2.003$ has been reported (Blubaugh et al., 1991; van Wijk et al., 1992). The ability to induce by light the the so-called S_2-state multiline EPR signal was also gradually lost during anaerobic photoinhibition (not shown) similarly to that observed after aerobic photoinhibition (Styring et al., 1990). It is also of note that no dark-stable S_2-state multiline signal was detected under conditions when triplet P_{680} formation was observed either in the presence or in the absence of the stable singly-reduced Q_A species (not shown). These results show that the donor side of the anaerobically photoinhibited triplet promoting PSII centers is in a largely reduced state.

DISCUSSION

We have recently observed that during strong anaerobic illumination the PSII centers undergo modifications which

Scheme I: Schematic Representation of the Proposed Sequential Reduction and Protonation Steps of Q_A Occurring during Anaerobic Photoinhibition



facilitate light-induced formation of $^3P_{680}$ (Vass et al., 1992). This phenomenon is of a great mechanistic importance since the formation of reaction center chlorophyll triplet in PSII is not yet understood in detail. In addition, singlet oxygen, which is formed via the interaction of $^3P_{680}$ and molecular oxygen, is a very reactive species whose damaging effects have been shown to trigger the D1 protein for degradation (Vass et al., 1992).

In the present work, we have extended our previous studies (Vass et al., 1992; Vass & Styring, 1992) by more detailed kinetic analysis of the formation and decay of $^3P_{680}$ promoting centers. In the following, we will analyze our results in the framework of a model for the stepwise reduction and protonation of Q_A during the course of strong illumination as summarized in Scheme I.

Single Reduction of Q_A. Shortly after the onset of the strong anaerobic illumination, PSII centers containing singly-reduced Q_A will dominate. Very early the $Q_A^-Fe^{2+}$ EPR signal decays rapidly (in few seconds), and this state in Scheme I is referred as $Q_A^-{}_{,norm}$. Most likely the dominating decay pathway for Q_A^- involves oxidized species on the donor side of PSII. These are the centers that were present early during photoinhibition, and in which Q_A could be reduced with dithionite in the dark leading to the incresed amplitude of the $Q_A^-Fe^{2+}$ EPR signal (Figure 2A). In centers with "normal Q_A^-", no light-induced $^3P_{680}$ formation can be observed.

Stabilization of Q_A^-. During the continued strong illumination, the singly reduced "normal" Q_A becomes stabilized as revealed by the formation of a more slowly decaying population of the $Q_A^-Fe^{2+}$ EPR signal ($t_{1/2} \approx 30$–35 min). The stabilization of Q_A^- is most likely caused by two factors: (i) the block of forward electron transport from Q_A^- to Q_B, which has been demonstrated by time-resolved fluorescence measurements in our previous work (Vass et al., 1992), and (ii) the absence of oxidized recombination partners at the donor side of PSII. This latter assumption is supported by the absence of the S_2-state multiline signal under the conditions where stable Q_A^- is present. The room-temperature stability of this Q_A^- population (30–35 min, Figure 4B) is higher than that obtained in the previous fluorescence study (2–3 min; Vass et al., 1992). The reason for this difference partly originates from the much higher chlorophyll concentration (1.5–2.5 mg of Chl/mL as compared to 20 μg of Chl/mL) used in the present study and/or from the more complete exclusion of oxygen from the EPR samples which is an important factor of Q_A^- reoxidation.

Protonation of Stable Q_A^-. The amount of centers with stabilized Q_A^- does not change at the beginning of the photoinhibitory illumination (Figure 2A). However, the fraction of $^3P_{680}$ forming centers is substantially increased in the same period (Figure 2B). In addition, the amount of triplet promoting centers increases faster than Q_A becomes doubly reduced. This is shown by the slower kinetics for the loss of the total $Q_A^-Fe^{2+}$ EPR signal as compared to the increase of the $^3P_{680}$ signal (Figure 2B). Thus, it is reasonable to suggest that the centers with stabilized Q_A^-, which initially do not promote $^3P_{680}$ formation, are gradually converted during further illumination to $^3P_{680}$ forming centers in which Q_A is still in the singly-reduced form (Scheme I). The amount of these centers increases fast during the early phase of photoinhibition, reaching transiently 25% of all centers (Figure 2B, dotted curve).

The absence (or very low yield) of $^3P_{680}$ formation which is usually observed when Q_A is singly reduced has been satisfactorily explained by the suppression of the primary radical pair formation due to electrostatic repulsion between Q_A^- and Phe^-. The electrostatic effect can be removed by the protonation of the reduced Q_A states or by the absence of Q_A (van Mieghem et al., 1989, 1992; Vass & Styring, 1992), leading to high yield of light-induced $^3P_{680}$. Our observation of high triplet yield in PSII centers which possess stable singly-reduced Q_A suggests that the charge on Q_A^- is neutralized. The formation of triplet promoting PSII centers is enhanced at low pH (Figure 3), and we propose that this reflects a protonation event at or near Q_A^- (Scheme I). This hypothesis is also supported by our previous fluorescence data showing a similar pH dependence of the increased F_0 fluorescence which arises from the centers which have stable Q_A^- (Vass et al., 1992).

Q_A^- in PSII can most likely become protonated below pH 8.9 under equilibrium conditions, as shown by the pH-dependent redox potential of the Q_A/Q_A^- redox couple (Knaff, 1975). However, Q_A^- does not seem to be protonated in intact PSII on the time scale of electron transport processes (Robinson & Crofts, 1984; Vass & Inoue, 1986). Thus, in the photoinhibited PSII centers the increased lifetime of Q_A^- (ranging up to tens of minutes) might allow also slow protonation reactions to take place. An alternative explanation is that a structural change exposes the Q_A binding region to the aqueous phase. A modified conformation of the photoinhibited PSII centers, which might be related to the protonation event, has already been suggested to occur (Vass et al., 1992).

When PSII samples in which $^3P_{680}$ is formed in the presence of stable reduced Q_A are incubated in darkness, a small population of the $Q_A^-Fe^{2+}$ signal (reflecting PSII centers with the "normal" form of Q_A^-; Scheme I) is lost within 1 min, while the rest decays with approximately a 30-min half-time (reflecting the "stable" Q_A^-; Scheme I). In the same samples most of the $^3P_{680}$ centers are lost within 2–3 min, while only a small fraction decays with about a 30-min half-time. A possible explanation for this complex behavior is that the proton which we propose to neutralize the negative charge on Q_A^- (or on a nearby base) is released faster in the dark (with $t_{1/2} \approx 2$–3 min) than the reoxidation of stable Q_A^- occurs (with $t_{1/2} \approx 30$–35 min).

Second Reduction and Protonation of $Q_A^-(H^+)$. The protonated form of Q_A^- is not very stable in the dark. Instead the proton at or near Q_A^- seems to be stabilized via the light-driven arrival of the second electron to form the state $Q_A^{2-}(H^+)$ during continued illumination under anerobiosis (Scheme I). This species is diamagnetic and results in the loss of the $Q_A^-Fe^{2+}$ EPR signal. The concept of electrostatic control of primary charge separation predicts the suppression of light-

induced $P_{680}^+Phe^-$, and consequently $^3P_{680}$, formation in the presence of a double-reduced and singly-protonated Q_A species, $Q_A^{2-}(H^+)$, similarly to the case of Q_A^- (Vass & Styring, 1992). However, the observation of very high $^3P_{680}$ yields shows that the second electron on Q_A is quickly neutralized, most likely by a second protonation (Scheme I). This results in the formation of reaction centers containing double-reduced and double-protonated Q_A ($Q_A^{2-}(H^+)_2$) which is quite stable and characterized by a slowly decaying ($t_{1/2} \approx$ 20–30 min) $^3P_{680}$ yield and by the absence of the $Q_A^-Fe^{2+}$ EPR signal. The formation of $Q_A^{2-}(H^+)$ as an intermediate to the $Q_A^{2-}(H^+)_2$ state might also be supported by the transient increase of $^3P_{680}$ formation observed during dark adaptation after a 12-min photoinhibition (Figure 4A). We suggest that this process reflects the decay of the non triplet promoting $Q_A^{2-}(H^+)$ to the triplet promoting $Q_A^-(H^+)$. An alternative explanation for the transient increase in the $^3P_{680}$ yield upon dark incubation of the 12-min sample could be the light-induced buildup and subsequent dark decay of reduced pheophytin. Reduced pheophytin would prevent charge separation and $^3P_{680}$ formation. However, this possibility can most likely be excluded, since there was no EPR-detectable Phe^- trapped under our experimental conditions (not shown).

Release of $Q_A(H_2)$. In PSII centers where no $Q_A^-Fe^{2+}$ can be observed, part of the $^3P_{680}$ forming population does not decay at all. We propose that in these centers the double-reduced and protonated Q_A has either left or been displaced from its proper binding site (Scheme I), similarly to what has been proposed to occur following chemical reduction of Q_A (van Mieghem et al., 1989). There also exists chemical evidence that Q_A might leave its site as a consequence of severe anaerobic photoinhibition (Koivuniemi et al., 1992).

Scheme I represents the summary of events that we propose to occur at the level of Q_A during anaerobic photoinhibition. An exact kinetic model of these events cannot be formulated at present, and further experiments to improve the kinetic resolution of the protonation/deprotonation steps are in progress. On the basis of the available data, Scheme I cannot be generalized for the events that take place during chemical double reduction of Q_A, and the possibility of other reaction sequences leading to $Q_A^{2-}(H^+)_2$ formation cannot be excluded.

Changes at the Donor Side of PSII. In chemically reduced PSII membranes of D1/D2 reaction center complexes, the ability of $^3P_{680}$ formation is apparently accompanied with the irreversible inactivation of the water-oxidizing complex. In contrast, in the anaerobically photoinhibited PSII membranes $^3P_{680}$ formation occurs in centers in which the donor side is only slightly modified. In all the three triplet promoting forms of the anaerobically photoinhibited PSII reaction center the water-oxidizing complex is trapped in the lower S-states (S_0 and S_1). Concomitant to the inhibition of the oxygen-evolving activity and the loss of the dark-stable $Q_A^-Fe^{2+}$ EPR signal, signal II_{slow} is also lost indicating the gradual reduction of Tyr_D^+. The mechanism of this process is not known at present, but one likely possibility involves a low-yield recombination between Tyr_D^+ and Phe^- in centers where P_{680}^+ is rereduced by a donor side redox component although Phe^- cannot be reoxidized via forward electron transfer when Q_A has become double reduced.

Magnetic Characteristics of the Different Triplet Forming PSII Centers. From our previous fluorescence and EPR study it was concluded that three different populations of photoinhibited PSII centers are able to promote $^3P_{680}$ formation (Vass et al.,1992). The present measurements of the $^3P_{680}$ EPR signal have demonstrated the existence of these three types of triplet promoting PSII centers. In all these three states the light-induced $^3P_{680}$ EPR signal has the same AEEAAE (A = enhanced absorption, E = emission) polarization pattern. This unique polarization pattern is identical to that observed in chemically reduced or highly purified PSII preparations or in purple bacteria (Budil & Thurnauer, 1991) and proves that $^3P_{680}$ in the anaerobically photoinhibited PSII centers is formed via the radical pair mechanism. In addition, the zero-field splitting parameters calculated from the triplet EPR spectra are the same in the different photoinhibited states, including those obtained in thylakoid membranes, as in the chemically reduced PSII centers (Table I). The identical magnetic parameters indicate that anaerobic photoinhibition and chemical double reduction of Q_A lead to the same modification of the PSII reaction center. The data of Table I also demonstrate that the triplet EPR signal of anaerobically photoinhibited thylakoids originates from PSII and not from PSI centers.

Table I: Zero-Field Splitting Parameters of PSI and PSII Reaction Center Triplets Compared to Anaerobically Photoinhibited PSII Membranes and Thylakoids

sample	$\|D\|$ ($cm^{-1} \times 10^4$)	$\|E\|$ ($cm^{-1} \times 10^4$)
PSII (D1/D2) reaction center particles	287[c]	43[c]
PSII membranes		
5 min anaerobic PI	287.2 ± 0.8[a]	44.2 ± 0.5[a]
20 min anaerobic PI	288.2 ± 0.6[a]	43.4 ± 0.5[a]
Chemical reduction	287.3 ± 0.5[a]	44.3 ± 0.4[a]
Chemical reduction	290[b]	44[b]
thylakoids	288.0 ± 0.6[a]	44.1 ± 0.6[a]
PSI membranes	283[b]	40[b]

[a] The zero-field splitting parameters were either calculated from the spin polarized EPR spectra measured in the present work (1) or taken from the literature. [b] Rutherford and Mullet (1981). [c] Okamura et al., (1987).

Relevance to Photoinhibition. Previously we have shwon that photoinhibition in PSII membranes results in the double reduction of Q_A (Styring et al., 1990; Vass et al., 1992). These conditions promote the degradation of D1 protein, probably via interaction of $^3P_{680}$ with oxygen resulting in singlet oxygen formation (Vass et al., 1992). The observation here, of light-induced buildup of $^3P_{680}$ promoting centers in the more intact thylakoid system, further supports the hypothesis that double reduction of Q_A, and consequently $^3P_{680}$ formation, applies to photoinhibition and D1 protein degradation also in vivo. In addition, the sequential reduction and protonation of Q_A provides an attractive molecular mechanism for the reactions that lead to double reduction of Q_A during photoinhibition.

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