Articles

Spectroscopic Characterization of Triplet Forming States in Photosystem II†

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ABSTRACT: Fluorescence and electron paramagnetic resonance (EPR) measurements have been applied to characterize chlorophyll triplet formation in the reaction center of photosystem II (PSII). A highly triplet forming state was generated in PSII membranes by chemical double reduction of the primary electron acceptor Q_A. In triplet forming PSII centers, the steady-state yield of chlorophyll fluorescence decreased to about 70% of the maximal fluorescence yield observed in closed PSII centers in which Q_A is singly reduced. The results are well interpreted in the framework of a model where the charge state of Q_A electrostatically controls the yield of primary charge separation [Schatz, G. H., Brock, H., & Holzwarth, A. R. (1988) *Biophys. J. 54*, 397–405]. Thus, high triplet yield and decreased, although still quite high, fluorescence indicate a charge-neutralized state of PSII in which Q_A is singly or doubly reduced and protonated or absent. The EPR signal of the triplet primary chlorophyll donor, $^3P_{680}$, is suppressed by illumination at 77 K concomitant with the formation of a cationic radical (g = 2.0025–2.0027, and 0.92 mT wide) that is stable in the dark. This is attributed to the oxidation of an accessory chlorophyll (Chl) in the vicinity of P_{680}. Electrostatic repulsion between Chl^+ and P_{680}^+ is likely to prevent primary charge separation, and in turn triplet formation, providing a further example of electrostatic control of primary charge separation. The triplet P_{680} EPR signal is also suppressed in the presence of oxygen. This effect, which is almost completely reversible by removing the oxygen, is attributed to the interaction of triplet P_{680} with triplet O_2.

The reaction center of photosystem II (PSII)[1] is composed of the D1 and D2 proteins that form a heterodimer (Nanba & Satoh, 1987) in strong structural and functional homology to the reaction center complex of purple bacteria [for recent reviews see Brudvig et al. (1989), Hansson and Wydrzynski (1990), and Andersson and Styring (1991)]. The D1/D2 heterodimer binds the redox cofactors required for the primary steps of light-induced electron transport: the primary chlorophyll electron donor, P_{680}, the first electron acceptor, Phe, and the primary, Q_A, and secondary, Q_B, quinone electron acceptors. The catalytic manganese cluster of the water-oxidizing complex is most likely also bound to the D1/D2 complex [see Andersson and Styring (1991)]. In addition to the bound redox components, each of the D1 and D2 proteins has a redox-active tyrosine residue: Tyr_Z, D1Tyr161 (Debus et al., 1988a) is the intermediate electron carrier between P_{680} and the Mn cluster; Tyr_D, D2Tyr161 (Debus et al., 1988b; Vermaas et al., 1988) is an auxiliary electron donor to P_{680}.

Excitation of the reaction center with light induces the formation of the primary radical pair, $P_{680}^+Phe^-$, within a few picoseconds. The charge separation is stabilized by rapid electron transfer from Phe^- to Q_A (200–300 ps) and by the slower rereduction of P_{680}^+ by Tyr_Z (50–250 ns) [see Rutherford (1988) and Hansson and Wydrzynski (1990)]. When the forward electron transfer from Phe^- to Q_A is blocked by either the absence or the double reduction of Q_A, the increased lifetime of $P_{680}^+Phe^-$ (25–40 ns; Takahashi et al., 1987; Crystall et al., 1989) allows spin dephasing and subsequent charge recombination of the radical pair transiently forming a spin-polarized triplet state of the primary donor [reviewed by Budil and Thurnauer (1991) and Miller and Brudvig (1991)].

The EPR spectrum of $^3P_{680}$ has the same AEEAAE (A = enhanced absorption, E = emission) polarization pattern as that of the corresponding triplet in purple bacteria. This shows that this triplet in PSII has the same origin as in purple bacteria and is formed via the radical pair mechanism. In the bacterial reaction center, high quantum yield of triplet formation is induced by single reduction of Q_A. Contrary to this, single reduction of Q_A in PSII does not promote the formation of $^3P_{680}$ with high yield which, however, has been observed after double reduction and subsequent protonation of Q_A (van Mieghem et al., 1989) or in reaction centers from which Q_A is lost (Okamura et al., 1987; Durrant et al., 1990). The low yield of $^3P_{680}$ formation in the presence of singly reduced Q_A has been suggested to reflect the suppression of the primary radical pair formation in PSII due to electrostatic repulsion between Phe^- and Q_A^- (van Mieghem et al., 1989; Schatz et al., 1987). Decreased yield of the $P_{680}^+Phe^-$ radical pair formation in the presence of Q_A^- is also supported by the increased yield of fluorescence in closed PSII reaction centers (i.e., when Q_A^- is present) relative to open PSII reaction centers

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[1] Abbreviations: Chl, chlorophyll; Mes, 4-morpholineethanesulfonic acid; P_{680}, primary electron donor of PSII; PSII, photosystem II; 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); Phe, pheophytin; $^3P_{680}$, triplet state of P_{680}.

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(i.e., when Q_A is present). If, however, the negative charge on singly or doubly reduced Q_A is neutralized, for example, by protonation, the fluorescence yield of PSII is expected to decrease relative to that of the closed state (van Mieghem et al., 1989), but so far this effect has not been experimentally demonstrated.

^{3}Chl in vitro and in antenna complexes is known to interact with molecular oxygen via intersystem crossing [see reviews by Cogdell and Frank (1987) and Asada and Takahashi (1987)]. This results in the formation of extremely reactive singlet oxygen that can damage pigments and proteins. The formation of singlet oxygen via this pathway would provide an attractive mechanism for light-induced damage to the D1 reaction center protein (Vass et al., 1992). Indeed, the lifetime of $^3P_{680}$ in isolated PSII reaction center complexes has been shown to be substantially shortened in the presence of oxygen (Durrant et al., 1990; Mathis et al., 1989) which indicates that one decay pathway involves $^3P_{680}$–$^3O_2$ interaction.

In recent studies of photoinhibition under anaerobic conditions, we have observed the buildup of centers that form the characteristic spin-polarized $^3P_{680}$ EPR signal with high yield during illumination (Vass et al., 1992). Under these conditions, the protonation of Q_A^- (Q_AH) and Q_A^{2-} (Q_AH_2) as well as the release of $Q_A(H_2)$ from its binding site has been shown to promote $^3P_{680}$ formation. In all of these triplet forming states of PSII, the fluorescence yield is intermediate between the low fluorescence observed in open PSII centers and the high fluorescence in closed PSII centers.

Here, we aim at a clarification of the fluorescence characteristics of triplet forming centers of PSII, and we also describe their interaction with oxygen in further detail. We demonstrate that the fluorescence yield from triplet forming PSII produced by chemical double reduction of Q_A is about 70% of that from closed PSII centers. It is also shown that the presence of oxygen reversibly decreases the formation of the $^3P_{680}$ EPR signal at 4 K.

Materials and Methods

Sample Preparation. PSII enriched membranes from spinach thylakoids were prepared according to the method of Berthold et al. (1981) with the modification described by Ono and Inoue (1985) and stored at −80 °C until use in a solution of 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 20 mM Mes, pH 6.3, at 3–4 mg of Chl/mL. For chemical double reduction of Q_A, PSII membranes were incubated anaerobically with 40 mM dithionite and 30 μM benzyl viologen at room temperature in darkness for different periods of time (up to 5 h) as described by van Mieghem et al. (1989).

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 a temperature controller. Data acquisition and data handling were performed with the ESP 300 program of the EPR spectrometer. For light-induced generation of the spin-polarized $^3P_{680}$ EPR signal, samples were illuminated with a 1000-W tungsten projector in the EPR cavity through a 5-cm-thick copper sulfate heat absorbing solution. The triplet spectra were obtained as light minus dark difference spectra.

Fluorescence Measurements. Steady-state chlorophyll *a* fluorescence was measured with a PAM fluorometer (Walz, Effeltrich, Germany) using the DA100 data acquisition program on a Victor 386A computer. Fluorescence measurements were performed at room temperature directly in the EPR tubes on the same samples as the EPR measurements. This permitted the direct comparison of fluorescence and EPR characteristics.

Results

We have recently demonstrated the buildup of $^3P_{680}$ forming PSII centers during anaerobic photoinhibition of PSII membranes (Vass et al., 1992). Three different states were found to promote $^3P_{680}$ formation at 4 K: centers in which Q_A^- is (i) protonated (Q_AH), (ii) double reduced and protonated (Q_AH_2), and (iii) lost from the PSII reaction center. Anaerobic photoinhibition is also accompanied with an increase of the dark F_0 fluorescence to a level lying between the F_0 and F_{max} fluorescence levels of nonphotoinhibited controls. This indicates that the fluorescence yield of triplet forming centers (Q_A is singly or doubly reduced and protonated or absent from its site) is intermediate between the fluorescence yield of open (Q_A is oxidized) and closed (Q_A is singly reduced) PSII centers.

To characterize the fluorescence properties of triplet forming centers in further detail, we applied chemical double reduction of Q_A which has been demonstrated to lead to quantitative $^3P_{680}$ formation (van Mieghem et al., 1989). Steady-state fluorescence and the EPR signals from $Q_A^-Fe^{2+}$ and $^3P_{680}$ were measured after each step of the reduction procedure. The results are summarized in Figure 1.

Initially, the samples were made anaerobic by flushing with argon. In the anaerobic samples, actinic light induced the usual increase of fluorescence yield from F_0 to F_{max} (Figure 1, panel A, curve a). This reflects the light-induced reduction of Q_A which is quickly reversed in the dark (not shown). The EPR signal arising from the interaction of Q_A^- with Fe^{2+} was absent in the dark but could be induced by illumination at 77 K which traps the single reduced state of Q_A (Figure 1, panel B, solid and dashed curves a, respectively). In agreement with earlier reports, there was no light-induced $^3P_{680}$ EPR signal observable at this stage (Figure 1, panel C, curve a).

In the second step of the reduction process, dithionite and benzyl viologen were added anaerobically to the samples. In the presence of the reductants, even after a short incubation time, the fluorescence yield was not increased by actinic illumination but stayed constant approximately at the level of F_0 of the control samples (Figure 1, panel A, curve b). The $Q_A^-Fe^{2+}$ EPR signal was fully developed and stable in the dark (Figure 1, panel B, curve b), indicating the complete single reduction of Q_A by dithionite. The light-induced $^3P_{680}$ EPR signal was still not observed at this stage of the reduction process (Figure 1, panel C, curve b). The surprisingly low fluorescence yield in the presence of fully reduced Q_A (Figure 1, panel B, curve b) is due to the strong fluorescence quenching by reduced benzyl viologen. No fluorescence quenching was observed by benzyl viologen in the absence of dithionite (not shown).

After dark incubation with dithionite and benzyl viologen for 5 h at room temperature, there was no further change in the fluorescence yield (Figure 1, panel A, curve c). However, the dark $Q_A^-Fe^{2+}$ EPR signal completely disappeared (Figure 1, panel B, curve c), indicating the formation of EPR silent double reduced Q_A [the $Q_A^-Fe^{2+}$ EPR signal could not be induced by 77 K illumination either (not shown)]. During illumination at 4 K, these samples showed a fully developed EPR signal from $^3P_{680}$ (Figure 1, panel C, curve c) in complete agreement with the results of van Mieghem et al. (1989).

At first sight, these results seem to indicate that the triplet forming state of PSII, in which Q_A is double reduced, has a rather low fluorescence yield. However, the situation is complicated by the presence of reduced benzyl viologen which is a potent fluorescence quencher. To reoxidize the reduced benzyl viologen, samples were flushed with oxygen; meanwhile the F_0 fluorescence was monitored. This treatment resulted

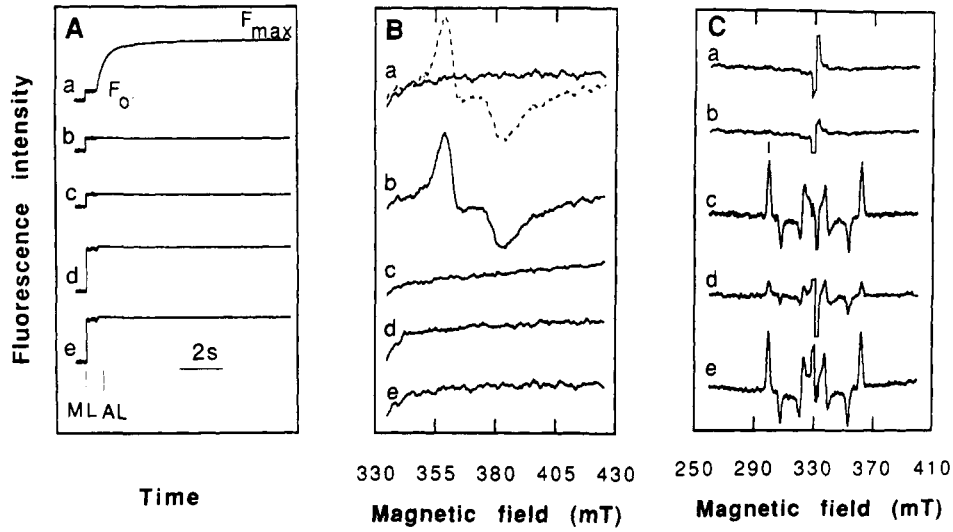

FIGURE 1: Changes of variable fluorescence and EPR characteristics during dithionite/benzyl viologen treatment of isolated PSII membranes. (A) Variable fluorescence. First, the F_0 fluorescence was detected with a weak measuring light, ML. This was followed by the measurement of the maximal F_{max} fluorescence induced by actinic light, AL, in each step of the experimental protocol: (a) after 3 min flushing with argon (anaerob control); (b) shortly after the addition of 50 mM dithionite and 30 μM benzyl viologen; (c) following 5 h of dark incubation of the PSII membranes with dithionite/benzyl viologen; (d) after 3 min of flushing with oxygen to reoxidize benzyl viologen; and (e) following 3 min of flushing with argon to remove the oxygen introduced in step d. (B) The $Q_A^-Fe^{2+}$ EPR signal. The EPR signal was measured after each different step (a–e) of the reduction procedure on the same samples as the variable fluorescence. The solid lines represent measurements performed on samples kept in darkness, whereas the dashed line a represents a measurement where Q_A^- was induced by 20 min of illumination at 77 K. EPR conditions: temperature, 4 K; microwave power, 32 mW; modulation amplitude, 3.2 mT. (C) The spin-polarized $^3P_{680}$ EPR signal. The triplet EPR signal was measured after each different step (a–e) of the reduction procedure on the same samples as the variable fluorescence and the $Q_A^-Fe^{2+}$ EPR signal. The central feature in the radical region of all spectra is a subtraction artifact due to a casual heating during illumination. The vertical bar indicates the field position where the decrease kinetics of the signal during 4 K illumination was measured. EPR conditions: temperature, 4 K; microwave power, 63 μW, modulation amplitude, 2.2 mT.

in an immediate increase of the F_0 fluorescence (not shown). The oxygen flush was continued for 3–5 min until no further increase of the F_0 fluorescence occurred. In the oxygen-flushed samples, the F_0 fluorescence level was substantially higher than in the control samples, and no further increase could be induced by actinic illumination (Figure 1, panel A, curve d). The $Q_A^-Fe^{2+}$ EPR signal was absent after the oxygen flush when it was measured in the dark (Figure 1, panel B, curve d) or after 77 K illumination (not shown). Oxygen flushing, however, also decreased substantially the size of the $^3P_{680}$ EPR signal (Figure 1, panel C, curve d).

The decrease of the $^3P_{680}$ EPR signal after the oxygen flush could indicate either the abolition of the triplet forming states (e.g., by reoxidation of double reduced Q_A) or a direct, oxygen-dependent, quenching of the $^3P_{680}$. To clarify the role of oxygen in the decreased triplet formation, the samples were again flushed with argon in the dark to remove the oxygen. The argon flush did not induce any further change, neither in the fluorescence yield (Figure 1, panel A, curve e) nor in the $Q_A^-Fe^{2+}$ EPR signal (Figure 1, panel B, curve e). The light-induced triplet formation, however, was almost completely restored (Figure 1, panel C, curve e). This result indicates that the oxygen flush did not destroy the triplet forming states in the dark. Taken together, the results in Figure 1 indicate that $^3P_{680}$ forming PSII centers give rise to high yield of constant fluorescence, F_0. This increased F_0 fluorescence yield is, however, smaller than the yield of F_{max} fluorescence observed in the presence of single reduced Q_A (compare curves a and d and e in Figure 1, panel A) in agreement with our previous findings obtained in the anaerobically photoinhibited PSII (Vass et al., 1992).

To confirm that the increased F_0 fluorescence and the ability to form a triplet in dithionite/benzyl viologen treated PSII membranes are really correlated, experiments similar to those in Figure 1A–C were performed after different reduction times. The $^3P_{680}$ EPR signal and the F_0 fluorescence, measured after

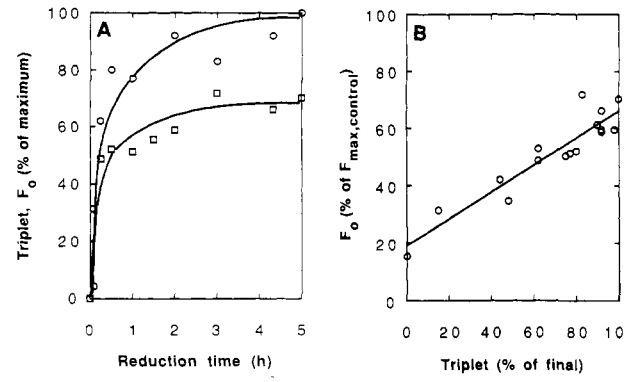

FIGURE 2: The buildup of the $^3P_{680}$ EPR signal and the increased F_0 fluorescence during dark chemical reduction of isolated PSII membranes. The samples were incubated with dithionite/benzyl viologen in the dark for different periods of time, and the F_0 fluorescence and the $^3P_{680}$ EPR signal were measured as in Figure 1. (A) The time course for the increase of the $^3P_{680}$ EPR signal (○) and the F_0 fluorescence (□). F_0 is plotted as a percentage of F_{max}. (B) The correlation of the F_0 fluorescence level and the size of the $^3P_{680}$ EPR signal.

oxygen and subsequent argon flushing of the samples to reoxidize benzyl viologen, increased in parallel with increasing dark incubation time (Figure 2A). The F_0 fluorescence reached about 70% of the maximal fluorescence yield of the nontreated control and increased in a linear correlation with the triplet size (Figure 2B).

During these experiments, we realized that subsequent EPR measurements on the same sample resulted in a decreasing size of the light-induced $^3P_{680}$ EPR signal at 4 K. The kinetics for this triplet quenching was studied in experiments performed at the field position of the first absorptive triplet peak (at 299 mT, Figure 1C, curve e). Switching on the light resulted in an immediate triplet formation which was followed by a biphasic decrease in the triplet amplitude (Figure 3, curve a). To rule out the possibility that sample heating during illu-

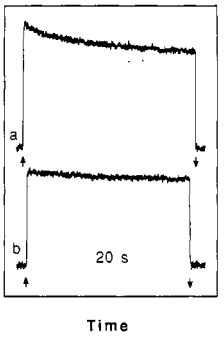

FIGURE 3: Kinetics of the decrease of the $^3P_{680}$ EPR signal during illumination at 4 K of chemically reduced PSII membranes. The kinetics of the triplet EPR signal was followed at the first low-field absorptive peak shown by the vertical bar on Figure 1C. The arrows indicate the switching on (↑) and off (↓) the illumination. The first (a) and the second (b) illumination period were separated by a dark period of 5 min.

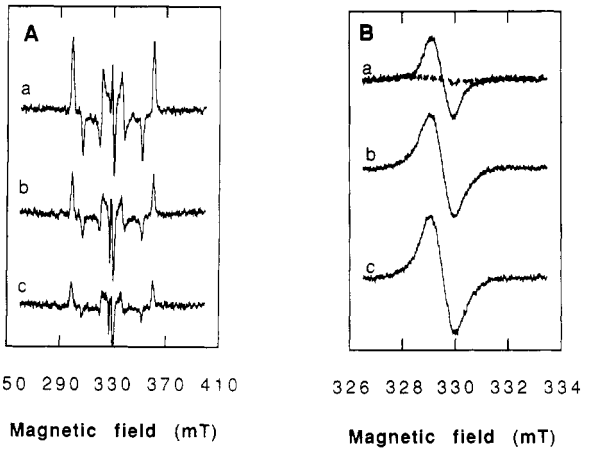

FIGURE 4: The effect of 77 K illumination of the $^3P_{680}$ (A) and the 0.92-mT-wide radical EPR signal (B) of chemically reduced PSII membranes. Samples were illuminated at 77 K for 1 min (a), 5 min (b), and 10 min (c) prior to EPR measurements. The dashed line in panel B, spectrum a, shows the radical region before the first illumination. EPR conditions for $^3P_{680}$ are the same as in Figure 1. EPR conditions for the radical signal: temperature, 4 K; microwave power, 63 nW; modulation amplitude, 0.3 mT.

mination was responsible for the initial fast decrease, a second illumination was applied following a dark period of 5 min. Indeed, the triplet induced by the second illumination had initially the same size as at the end of the first illumination period and decreased slowly further (Figure 3, curve b). A similar light-induced decrease was also observed on the much smaller triplet of the oxygenated samples (not shown). It is important to note that the loss of the $^3P_{680}$ EPR signal during illumination was accompanied with the induction of a radical signal which is described in detail below.

The triplet decrease observed during the 4 K illumination suggests the light-induced formation of a triplet quencher. This might be related to or identical with the species that gives rise to the radical EPR signal. At 4 K, the triplet decrease and the radical formation is rather small, probably due to the limited formation of the triplet quencher. Therefore, we investigated the effect of 77 K preillumination of reduced samples on the triplet formation measured subsequently at 4 K. As shown in Figure 4A, illumination at 77 K induced a substantial loss of the $^3P_{680}$ EPR signal measured at 4 K. In parallel with the triplet decrease, a radical EPR signal was induced (Figure 4B). The radical is the same as induced during the prolonged 4 K illumination: it is 0.92–0.93 mT wide and has a g value of 2.0025–2.0027 and is lost during 5 min

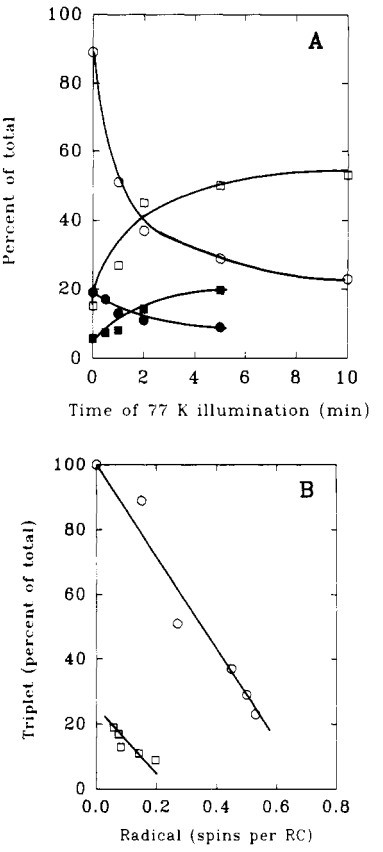

FIGURE 5: Changes in the size of the $^3P_{680}$ and the 0.92-mT-wide radical EPR signals during 77 K illumination. Chemically reduced PSII membranes were preilluminated at 77 K for different periods of time, and the $^3P_{680}$ and radical EPR signals were measured as in Figure 4. (A) The time course of the $^3P_{680}$ and the 0.92-mT-wide radical EPR signals during 77 K illumination. The size of the $^3P_{680}$ signal in the anaerobic (○) and oxygen-flushed samples (●) is shown as a percentage of the initial signal size in anaerobic samples. The less than 100% triplet size at 0 min of 77 K illumination reflects the decrease induced by the 4 K illumination during the recording of the first triplet spectrum (3 min of illumination at 4 K). The intensity of the radical signal in the anaerobic (□) and oxygen-flushed samples (■) is obtained by double integration and is shown relative to the intensity of signal II_{slow} from Tyr_D of untreated controls. The radical size at 0 min of 77 K illumination is obtained after the measurement of the $^3P_{680}$ EPR signal induced by 4 K illumination. (B) The correlation of the $^3P_{680}$ and the radical signals in the anaerobic (○) and oxygen-flushed samples (□).

of dark incubation at room temperature (not shown). The time course of the triplet decrease and the radical induction during the 77 K illumination is shown in Figure 5A. A 10-min illumination at 77 K resulted in an 80% decrease in triplet yield concomitant with a large radical induction. After 10 min, the radical signal amounted approximately to 0.5–0.6 spin per PSII reaction center as quantified on the basis of signal II_{slow} arising from Tyr_D^+ in control samples. A similar effect was also observed after oxygen flush of the samples although to a much smaller extent (Figure 5A). The light-induced decrease of the $^3P_{680}$ EPR signal and the induction of the 0.92-mT-wide radical show a linear correlation both after preillumination at 77 K (Figure 5B) and 4 K (not shown).

Discussion

Fluorescence Yield of Triplet Forming PSII Centers. Fluorescence yield as well as triplet formation in PSII is strongly influenced by the redox state of Q_A. A kinetic and energetic scheme of these effects, based on the works of Schatz et al. (1988) and van Mieghem et al. (1989), is presented in Figure 6.

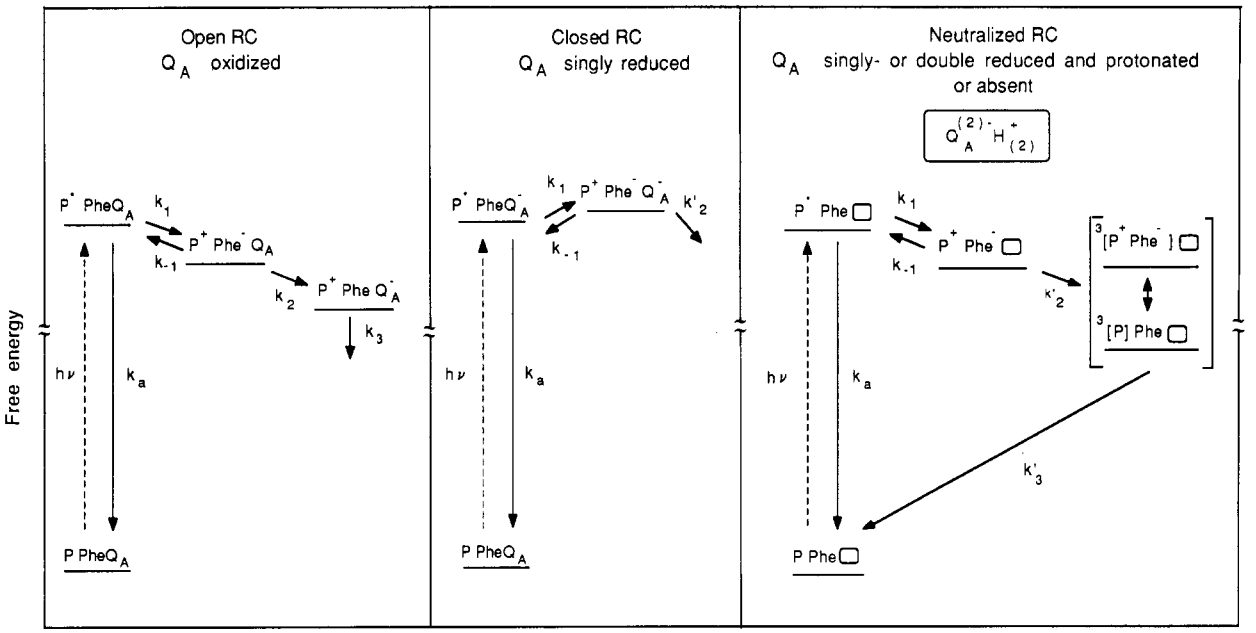

FIGURE 6: Simplified schemes of free energy levels and primary electron-transfer steps in the open, closed, and neutralized states of the PSII reaction center. In the open and closed state, Q_A is oxidized and singly reduced, respectively. In the neutralized state, Q_A is either singly or doubly reduced and protonated or missing from the binding site. In this scheme, the rate constants k_i describe $k_a = k_d + k_{rad}$, the radiationless plus the radiative decay of excited P_{680} (P) and the equilibrated antenna chlorophylls: k_1, the apparent charge separation; k_{-1}, recombination of the primary charge pair to the excited state; k_2, reoxidation of Phe^- by Q_A; k_3, charge stabilization associated with the rereduction of P_{680}^+; k_2', the process of spin dephasing and $^3P_{680}$ formation; k_3', the decay of $^3P_{680}$.

In the open state of the reaction center, i.e., when Q_A is oxidized, the primary charge separation is energetically favorable, leading to a high rate of $P_{680}^+Phe^-$ formation. Due to the efficient charge separation, the lifetime of the excitation in the reaction center and the closely coupled antenna chlorophylls is short. This results in a low fluorescence yield (F_0). The separated charge pair is stabilized by fast electron transfer from Phe^- to Q_A. Due to this fast forward reaction, the lifetime of the primary charge pair is too short to allow spin dephasing which thereby prevents the formation of $^3P_{680}$.

Upon reduction of Q_A, the yield of chlorophyll fluorescence increases from the F_0 level to the F_{max} level that is 4 to 5 times higher than F_0 in isolated thylakoids or PSII membranes. The most likely cause for this effect is an electrostatic repulsion between the closely lying Q_A^- and Phe^- (approximately 13 Å), which was suggested to make $P_{680}^+Phe^-$ formation slightly endothermic (Schatz et al., 1988). In these closed reaction centers, the inefficient charge separation increases the lifetime of excited chlorophylls which consequently increases the fluorescence yield. This idea is well supported by time-resolved fluorescence (Schatz & Holzwarth, 1986; Schatz et al., 1987), absorption change (Schatz et al., 1987), and photovoltage measurements (Trissl et al., 1987). The decreased yield of the primary charge separation in the presence of Q_A^- was suggested to be the cause also for the lack of triplet formation in PSII with single reduced Q_A. Our EPR measurements show that the yield of $^3P_{680}$ formation in the presence of Q_A^- is less than 1–2% of that observed after chemical double reduction of Q_A.

The high triplet yield in PSII observed after double reduction of Q_A or in purified reaction center complexes was explained by charge neutralization by protonation or by the absence of Q_A. Both of these events remove the electrostatic effect and restore efficient $P_{680}^+Phe^-$ formation (van Mieghem et al., 1989). In the charge-neutralized state of PSII, approximately the same rate and yield of $P_{680}^+Phe^-$ formation is expected as in open reaction centers. However, in the absence of forward electron transport from Phe^- to Q_A, the $P_{680}^+Phe^-$ radical pair is relatively long lived. The extended lifetime of $P_{680}^+Phe^-$ allows spin dephasing to form $^3[P_{680}^+Phe^-]$, which subsequently recombines to the $^3P_{680}$ state.

From the efficient charge separation and the blocked forward electron transfer, the neutralized reaction center is expected to behave like an open center with an increased fluorescence yield. This proposal has been confirmed in the present study. It was observed that the increase in the F_0 fluorescence yield showed a linear correlation with the amount of triplet forming centers produced by dark chemical double reduction of Q_A (Figure 2A,B). When all PSII centers were converted to neutralized, triplet forming centers, the F_0 fluorescence reached about 70% of F_{max} in the untreated control. This also confirms our earlier observation of an increased F_0 fluorescence in triplet forming centers of anaerobically photoinhibited PSII membranes.

To investigate whether our experimental results were compatible with the scheme presented in Figure 6, we performed model calculations using the mathematical formalism of Schatz et al. (1988) to estimate triplet and fluorescence yields in the charge-neutralized state of PSII. For the open and closed reaction centers, the rate constants were taken from Schatz et al. (1988). For the charge-neutralized state, where electrostatic effects do not influence the rates of formation and recombination of the primary charge pair, we used the same k_1 and k_{-1} values as for the open state. k_2' was estimated as 0.05–0.1 ns^{-1} from the rate of carotenoid triplet formation (Takahashi et al., 1987), which occurs most likely via $^3P_{680}$ (Booth et al., 1990), and from the rate of spin dephasing of the primary charge pair in purple bacteria (Hoff, 1987; van Wijk & Schaafsma, 1988). From the room temperature decay rate of $^3P_{680}$ (Durrant et al., 1990) k_3' was estimated as 1 ms^{-1}. The results from the model calculation are presented in Table I. These calculations predict a 4.5–5.0 times increase of the fluorescence yield in the closed centers relative to the open centers in good agreement with the experimental results. In the charge-neutralized state, the calculated fluorescence yield was 86–92% of that in the closed state, whereas the expected

Table I: Estimated and Measured Fluorescence and Triplet Yields in the Open, Closed, and Neutralized States of the PSII Reaction Center[a]

	open	closed	neutralized
k_a (ns^{-1})	0.9	1.1	0.9
k_1 (ns^{-1})	9.3	1.5	9.3
k_{-1} (ns^{-1})	2	2.4	2
k_2 (ns^{-1})	2		
k_2' (ns^{-1})		0.05–0.1	0.05–0.1
$\Phi_{fl}/\Phi_{fl,closed;calcd}$	0.20–0.21	1.0	0.94–0.86
$\Phi_{fl}/\Phi_{fl,closed;exptl}$	0.18–0.20[b]		0.7–0.75[b]
$\Phi_{triplet;calcd}$		0.03–0.05	0.26–0.33
$\Phi_{triplet;exptl}$		<0.01–0.02[c]	0.23–0.3[d]

[a] The fluorescence and triplet yields were calculated by using the reaction scheme in Figure 6 and the mathematical model of Schatz et al. (1988). [b] Experimental data are taken from the present work, measured on chemically reduced PSII membranes at room temperature. [c] Experimental data are taken from the present work, measured on chemically reduced PSII membranes at 4 K. [d] Experimental data are taken from the works of Takashashi et al. (1987) and Durrant et al. (1990) performed on D1/D2 particles at room temperature.

triplet yield varied between 26 and 33% at room temperature, where these calculations are valid. The predicted fluorescence yields are in a good quantitative agreement with our measurements (Figure 1, panel A).

The triplet yield at room temperature in our chemically reduced PSII membranes has not been determined yet. However, using experimental results obtained with D1/D2 particles, it is possible to derive a reasonable value for the triplet yield at room temperature. At 9 K, the triplet yield in double reduced PSII membranes has been shown to be close to 100% (van Mieghem et al., 1989). This is very similar to the about 80% triplet yield observed at 10 K in D1/D2 particles, which lack Q_A (Takahashi et al., 1987). At room temperature, the triplet yield in D1/D2 particles decreases to 23–30% (Takahashi et al., 1987; Durrant et al., 1990). If the temperature dependence of triplet formation in the reduced PSII membranes is similar, then the room temperature triplet yield would also be in excellent agreement with the predicted data in Table I calculated on the basis of room temperature rate constants.

Our results demonstrate that the earlier suggested concept of electrostatic control of the primary charge separation (Schatz et al., 1987, 1988) can be extended from the open and closed states to the state of PSII where the charge on Q_A is neutralized. Thus, this model provides a useful framework to interpret the changes in fluorescence and triplet yields observed upon reduction and charge neutralization of Q_A. A very important test of the validity of the model will be provided by parallel measurements of the yield of $^3P_{680}$ and fluorescence at room temperature.

It is also likely that the model will provide a sound theoretical basis for the events that occur at the acceptor side of PSII as a consequence of strong illumination (so-called photoinhibition). When photoinhibition is performed under anaerobic conditions, it results in the increase of the F_o fluorescence. Initially, the F_o increase is due to accumulation of singly reduced Q_A in which state PSII is not triplet forming. However, it has recently been shown (Vass et al., 1992) that the strong illumination subsequently leads to the formation of a state of PSII in which both Q_A^- and $^3P_{680}$ can be detected. From experiments carried out at different pH's, it was concluded that Q_A in these PSII centers was singly reduced and protonated. In those PSII centers, the F_o fluorescence was significantly higher than in the open centers but was somewhat lower than in the closed centers. This is very similar to the present results obtained with chemically reduced samples, and we believe that the theoretical model will be fruitful also to explain the results in PSII where Q_A is singly reduced and protonated (Figure 6). The quantitative results from anaerobic photoinhibition experiments designed to test this hypothesis will be published elsewhere.

Loss of $^3P_{680}$ Formation and Induction of a 0.92-mT Radical. Electrostatic control of the primary charge separation in principle should not be limited to the repulsive interaction of Q_A^- and Phe^-. A similar effect is expected if a positively charged species, like oxidized chlorophyll or carotenoid, were present close to P_{680}. Our data (Figures 4 and 5) demonstrate a new phenomenon of gradually decreasing triplet formation by illumination at 77 K (or 4 K). The decreased triplet formation is accompanied with the induction of a 0.92–0.93-mT-wide, g = 2.0025–2.0027, radical EPR signal. The EPR parameters of this radical are close to but not exactly the same as those reported for a PSII Chl^+ radical (g = 2.0026, 1.0–1.1 mT wide) or P_{680}^+ (g = 2.0026, 0.8 mT wide) and are not totally dissimilar from the Phe^- (g = 2.0035, 1.25 mT wide) radical. If we judge from the EPR parameters, it is likely that the radical we observe is a cationic radical originating from an oxidized chlorophyll which is slowly formed during illumination. How then could the presence of this radical prevent the formation of $^3P_{680}$? We see two possible mechanisms for this: (i) The radical represents the oxidized form of the chlorophyll species that gives rise to the spin-polarized triplet; (ii) the oxidized chlorophyll is located close to P_{680}, thereby preventing the charge separation via electrostatic control.

What happens then to Phe^- in the centers? It obviously does not recombine with the donor side, since the presumed cationic radical is stable for an extended period of time. Phe^- itself does not persist since we cannot detect it by EPR. It could possibly be reoxidized by oxygen. However, this should probably result in a much bigger radical formation in the oxygen-flushed than in the strictly anaerobic samples. This is opposite to what was observed (Figure 5). Thus, Phe^- might be reoxidized via an exogenous, EPR-silent species. As an alternative explanation, the radical signal we observe is a composite of a Chl^+ and Phe^- radical.

Triplet Quenching by Oxygen. Chlorophyll triplet states either in vitro or in the antenna complexes are known to interact with molecular oxygen that quenches the triplet in a reaction that leads to singlet oxygen formation:

$$^3Chl + {}^3O_2 \rightarrow Chl + {}^1O_2$$

The produced singlet oxygen is a very reactive species that might damage pigments and proteins. Triplet mediated formation of singlet oxygen in the reaction center of PSII would provide an attractive, chemically well understood, mechanism for the light-induced damage to the D1 reaction center protein during photoinhibition. This is supported by our earlier result showing that $^3P_{680}$ forming centers are irreversibly lost during illumination in the presence of oxygen concomitant with the degradation of the D1 protein (Vass et al., 1992). In agreement with this, the lifetime of $^3P_{680}$ detected optically in D1/D2 particles was shown to decrease from 1 ms to 30 μs in the presence of oxygen (Durrant et al., 1990). In the present study, we have observed an extensive quenching of the $^3P_{680}$ EPR signal by oxygen in the dark in PSII with double reduced Q_A. This effect, which is almost completely reversed by removing the oxygen from the samples, can be explained by the following alternatives: (i) $^3P_{680}$ is quenched via direct interaction with $^3O_2$ which produces P_{680} and $^1O_2$. This process is known to occur at room temperature as shown by optical measurements in D1/D2 reaction center complexes (Durrant et al., 1990). Direct triplet quenching by oxygen most likely

takes place at cryogenic temperatures as well. However, its efficiency should be limited by the availability of oxygen molecules in the vicinity of $^3P_{680}$ and by the rate of restoring $^3O_2$ from $^1O_2$ at cryogenic temperatures.

(ii) The presence of oxygen could accelerate the decay of the triplet state and thereby decrease the size of the steady-state EPR signal by enhancing spin-lattice relaxation, caused by the interaction of $^3P_{680}$ with vibrational states of the surrounding protein. In this case, the $^3P_{680}$ EPR signal, detectable in the presence of oxygen, is expected to become asymmetric as revealed by the decrease of the emissive transitions relative to the absorptive ones (Kleibeuker & Schaafsma, 1974; Searle et al., 1990). Indeed, a small asymmetry of this type can be observed in the EPR signal of the oxygen-flushed samples (Figure 1, panel C, spectrum d).

(iii) Oxygen accepts electrons from Phe^-, thereby destabilizing the $P_{680}^+Phe^-$ charge pair. In this case, accumulation of a radical, either P_{680}^+ or an accessory Chl^+, during illumination would be expected. However, our EPR measurements showed no light-induced formation of a radical at 4 K in the oxygen-flushed samples which could account for the loss of about 80% of $^3P_{680}$.

The experimental and literature data seem to support the first alternative. However, beside the direct interaction of $^3P_{680}$ with $^3O_2$ an additional effect of enhanced spin-lattice relaxation cannot be excluded in the reversible quenching of EPR signal by oxygen at cryogenic temperatures.

Conclusions

The fluorescence and EPR data presented here are in a good qualitative agreement with the earlier proposed models of electrostatic control of primary charge separation by the redox (charge) state of Q_A, which in turn influences fluorescence and triplet yields. In addition, the results in this paper have permitted the extension of the already existing concept for the charge-neutralized states, thereby improving the experimental support for the model. The light-induced formation of a cationic radical, likely an oxidized chlorophyll, which suppresses the $^3P_{680}$ EPR signal may indicate the existence of another form of electrostatic control of the primary charge separation, manifested via repulsion between Chl^+ and P_{680}^+. The reversible quenching of the $^3P_{680}$ EPR signal by oxygen can be attributed mainly to a direct interaction between $^3P_{680}$ and $^3O_2$ at cryogenic temperatures.

Registry No. P_{680}, 53808-91-6; O_2, 7782-44-7.

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