Electron Transfer in Photosystem II at Cryogenic Temperatures†

Julio C. de Paula, Jennifer B. Innes, and Gary W. Brudvig*
Department of Chemistry, Yale University, New Haven, Connecticut 06511
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ABSTRACT: The photochemistry in photosystem II of spinach has been characterized by electron paramagnetic resonance (EPR) spectroscopy in the temperature range of 77–235 K, and the yields of the photooxidized species have been determined by integration of their EPR signals. In samples treated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a single stable charge separation occurred throughout the temperature range studied as reflected by the constant yield of the Fe(II)–Q_A^- EPR signal. Three distinct electron donation pathways were observed, however. Below 100 K, one molecule of cytochrome b_{559} was photooxidized per reaction center. Between 100 and 200 K, cytochrome b_{559} and the S_1 state competed for electron donation to $P680^+$. Photooxidation of the S_1 state occurred via two intermediates: the $g = 4.1$ EPR signal species first reported by Casey and Sauer [Casey, J. L., & Sauer, K. (1984) *Biochim. Biophys. Acta 767*, 21–28] was photooxidized between 100 and 160 K, and upon being warmed to 200 K in the dark, this EPR signal yielded the multiline EPR signal associated with the S_2 state. Only the S_1 state donated electrons to $P680^+$ at 200 K or above, giving rise to the light-induced S_2-state multiline EPR signal. These results demonstrate that the maximum S_2-state multiline EPR signal accounts for 100% of the reaction center concentration. In samples where electron donation from cytochrome b_{559} was prevented by chemical oxidation, illumination at 77 K produced a radical, probably a chlorophyll cation, which accounted for 95% of the reaction center concentration. This electron donor competed with the S_1 state for electron donation to $P680^+$ below 100 K. Chemical oxidation of cytochrome b_{559}, however, had no effect on the photooxidation of the $g = 4.1$ or multiline EPR signal species. Quantitation of the cytochrome b_{559} EPR signal produced by chemical oxidation showed that two molecules of cytochrome b_{559} are present per reaction center. The $S_2 \rightarrow S_3$ transition occurred in samples illuminated above 190 K. The $g = 4.1$ EPR signal was not detected, however, as an intermediate in the $S_2 \rightarrow S_3$ transition. We propose that the $g = 4.1$ and multiline EPR signals both arise from the same site in the S_2 oxidation state and the spectroscopic differences reflect temperature-dependent structural changes in the Mn active site.

The photooxidation of H_2O to O_2 in plants, algae, and cyanobacteria is catalyzed by the O_2-evolving complex (OEC)[1] of photosystem II (PSII). The four oxidizing equivalents necessary for this process are created by successive charge separations in the PSII reaction center and stored stepwise in the OEC, which can exist in five intermediate oxidation states called S_i ($i = 0$–4) states (Kok et al., 1970). The S_0 and S_1 states are dark-stable and present in a ratio of 1:3 in short-term dark-adapted thylakoid and PSII membranes (Forbush et al., 1971). Studies by Velthuys & Visser (1975), Vermaas et al. (1984), and Hanssum et al. (1985) indicate that long-term dark incubation changes the S_0:S_1 ratio to essentially 0:1. The mechanism for this process is not known, but it may involve reverse turnover of the OEC (Beck et al., 1985).

The identities of the redox active sites are slowly being unraveled. The involvement of manganese in O_2 evolution [for a review, see Amesz (1983)] and the discovery by Dismukes & Siderer (1981) of a multiline S_2-state EPR signal, which is characteristic of an exchange-coupled manganese complex, suggest that the catalytic site the OEC may consist of as many as four manganese ions in close magnetic interaction.

There is currently a great interest in defining the sequence of electron-transfer steps that occurs on the donor side of PSII and, in addition, in determining the structure and function of the various intermediates. EPR spectroscopy has been shown to be an especially powerful method for the identification of electron carriers and the determination of the sequential electron-transfer reactions and/or structural changes that occur during S-state advancement.

It is possible to monitor the $S_1 \rightarrow S_2$ state transition by EPR spectroscopy following illumination of PSII membranes at cryogenic temperatures. Illumination at 200 K produces the multiline EPR signal characteristic of the final oxidation product in the S_2 state (Brudvig et al., 1983). More recently, two laboratories (Casey & Sauer, 1984; Zimmerman & Rutherford, 1984) have reported a broad, light-induced EPR signal centered at $g = 4.1$, which was also associated with O_2 evolution. Casey and Sauer generated the $g = 4.1$ EPR signal by illuminating dark-adapted PSII membranes at 140 K. Since this EPR signal disappeared upon warming to 190 K in darkness to yield the S_2-state multiline EPR signal, the researchers assigned the $g = 4.1$ EPR signal to a precursor of the S_2 state. Zimmerman & Rutherford (1984), however, produced the $g = 4.1$ and multiline EPR signals concurrently by illumination at 200 K. In samples where double oxidation of the OEC was prevented by treatment with DCMU, illumination at 200 K produced only the S_2-state multiline EPR signal, suggesting that the species giving rise to the $g = 4.1$ EPR signal was an intermediate between the S_2 and S_3 states. It was proposed (Zimmerman & Rutherford, 1984) that the $g = 4.1$ EPR signal arises from an electron carrier that mediates electron transfer between the Mn site and $P680^+$ in both

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[1] Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting enzyme Y; chl, chlorophyll; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; OEC, oxygen-evolving complex; P680, primary electron donor in PSII; PSII, photosystem II; Z, endogenous electron donor to P680.

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the $S_1 \rightarrow S_2$ and the $S_2 \rightarrow S_3$ transitions.

The photooxidation of several other components of PSII at cryogenic temperatures has been reported. These include the EPR signal II species (Nugent et al., 1982), which has been assigned to a quinone cation radical (O'Malley et al., 1984), cytochrome b_{559} (Vermeglio & Mathis, 1975; Crowder et al., 1982), and a 10 G wide free radical EPR signal, which has been tentatively assigned to a carotenoid (Nugent et al., 1982) or chlorophyll (Visser & Rijgersberg, 1975) cation radical. Neither cytochrome b_{559} nor a carotenoid species, however, has been observed to act as an electron donor to $P680^+$ under conditions where O_2 evolution occurs, and their photooxidation at low temperature may not reflect a physiological role of these two species.

In this paper, we report the photochemical events that take place in PSII between 77 and 235 K. Our objective is to quantitatively account for charge separation in one and two electron transfer reactions by monitoring the various electron donor and acceptor species in PSII by EPR spectroscopy.

Experimental Procedures

The procedure for isolation of PSII membranes and production of the S_2-state multiline EPR signal has been described by Beck et al. (1985). The chlorophyll concentration in the EPR samples ranged from 3.0 to 6.0 mg of chl/mL. PSII preparations used in this study exhibited rates of O_2 evolution between 300 and 500 μmol of O_2 (mg of chl)$^{-1}$ h^{-1} when assayed in a medium containing 2,5-dichloro-*p*-benzoquinone (DCBQ) as an electron acceptor.

After dark adaptation for 4 h at 0 °C, the samples were illuminated at 190 K for 2 min, warmed to 0 °C for 30 min in the dark, and then frozen at 77 K. This procedure ensured that most if not all of the centers were initially in the S_1 state (Goodin et al., 1984). The investigation of one electron transfer reactions was carried out in samples that were treated with enough 3 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in 95% ethanol to give a final DCMU concentration of 50 μM. Samples in which two electron transfer reactions were studied contained approximately a 10-fold excess of DCBQ over the reaction center concentration. Chemical oxidation of cytochrome b_{559} was achieved by treating PSII membrane samples with 2 mM K_2IrCl_6 (Aldrich), incubating for 5–15 min in the dark, and then freezing at 77 K.

The spin quantitation procedure described by Aasa & Vänngård (1975) was used for estimating the concentration of cytochrome b_{559} from its $g = 3.0$ turning point in the first derivative EPR spectrum. The spin standard was metmyoglobin azide, prepared according to Bolard & Garnier (1972) from sperm whale myoglobin (Sigma, type II, 95–100% purity) and NaN_3 (Mallinckrodt, practical). The ratio of chl to reaction center was determined by the EPR signal II spin quantitation procedure described by Babcock et al. (1983), with potassium nitrosodisulfonate (Aldrich) as a standard. In the PSII preparations used in this study, we have obtained values ranging from 120 to 170 chl/PSII.

Results

One Electron Transfer Reactions in PSII. In order to investigate single electron transfer events in PSII, we have added 50 μM DCMU to dark-adapted PSII membrane samples. DCMU is known to prevent the transfer of electrons between the quinone acceptors Q_A and Q_B by binding tightly to the Q_B site (Joliot & Kok, 1975), thereby limiting PSII to a single-charge separation.

Figure 1 shows the effect of illumination at three different temperatures on the EPR spectrum of dark-adapted PSII membranes. At 77 K (Figure 1a), cytochrome b_{559}, with

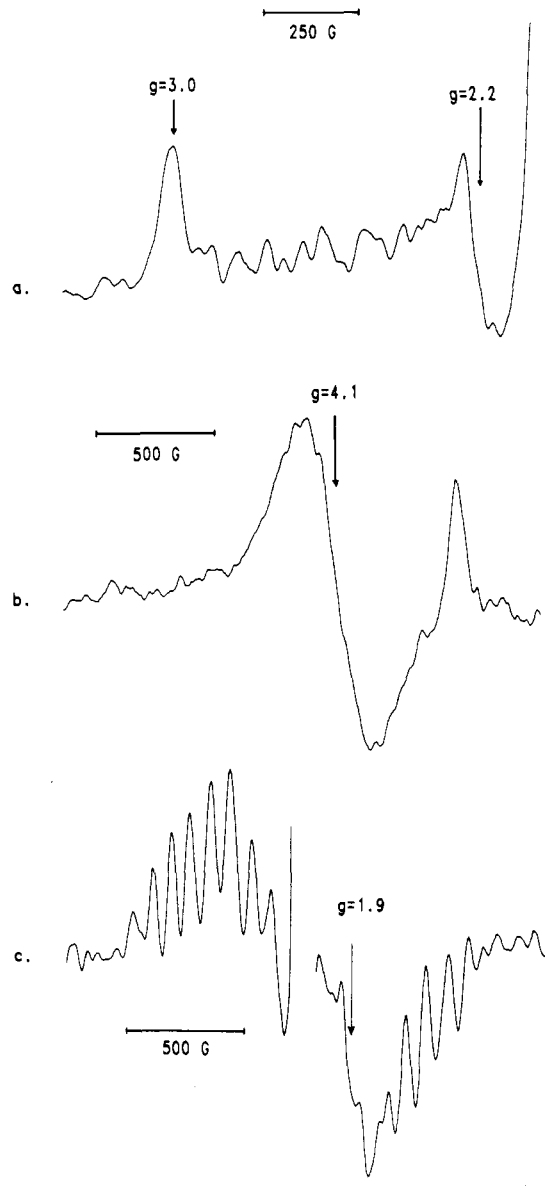


FIGURE 1: EPR signals produced by low-temperature illumination of 4-h dark-adapted PSII membranes: (a) 77 K illuminated minus dark; (b) 130 K illuminated minus dark; (c) 200 K illuminated minus dark. Instrument conditions: microwave frequency 8.81 GHz, microwave power 0.2 mW, modulation frequency 100 kHz, modulation amplitude 20 G, and sample temperature 10 K.

turning points at $g = 3.0$, $g = 2.2$, and a third estimated to be at $g = 1.3$ (Crowder et al., 1982), was photooxidized. At 130 K (Figure 1b), the light-induced $g = 4.1$ EPR signal first reported by Casey & Sauer (1984) was observed, along with some photooxidized cytochrome b_{559}. At 200 K (Figure 1c), the multiline EPR signal discovered by Dismukes & Siderer (1981) and associated with the S_2 state of the OEC was produced. Also apparent in Figure 1c is the EPR signal at $g = 1.9$, which arises from the reduced Q_A acceptor magnetically coupled to a ferrous ion (Nugent et al., 1981; Rutherford & Mathis, 1983). The intensity of the Fe(II)–Q_A^- EPR signal was identical for all three illumination temperatures, suggesting that all of the light-induced EPR signals discussed above derive from one stable charge separation in PSII.

It is possible to quantitate the cytochrome b_{559} EPR signal by using the method of Aasa & Vänngård (1975), which estimates the total integrated area from the area of the turning point at $g = 3.0$ in the first derivative spectrum. Figure 2

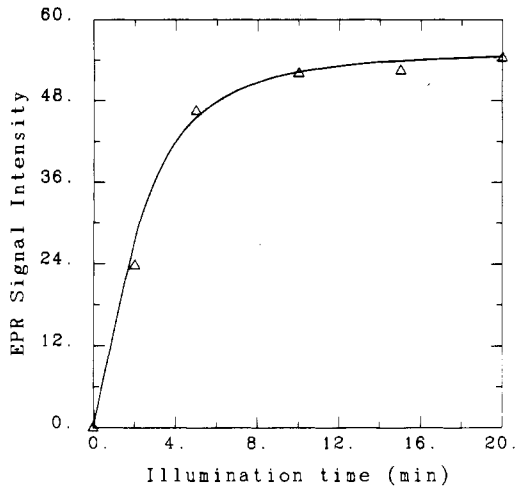


FIGURE 2: Effect of illumination at 77 K of 4-h dark-adapted PSII membranes treated with 50 μM DCMU on the EPR signal associated with oxidized cytochrome b_{559}. Instrument conditions were as Figure 1. Signal intensities were determined as the peak to base-line height of the peak at g = 3.0.

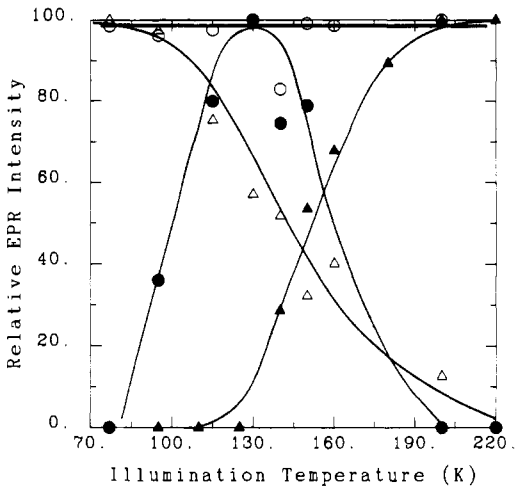


FIGURE 3: Effect of illumination on the signal amplitudes of the g = 4.1 (full circles), multiline (full triangles), cytochrome b_{559} (open triangles), and Fe(II)–Q_A^- (open circles) EPR signals. Signal intensities were determined as peak to peak heights; for the multiline signal, three peaks to high field and three peaks to low field of g = 2.0 were added together; for the cytochrome b_{559} signal, data were analyzed as in Figure 2. The intensity of each EPR signal was normalized with respect to its maximum yield. Instrument conditions were as in Figure 1.

shows that after 10 min of illumination at 77 K the intensity of the EPR spectrum of oxidized cytochrome b_{559} reached a maximum. By integrating the g = 3.0 turning point, in order to obtain the concentration of cytochrome b_{559}, and determining the concentration of chl in the sample (Arnon, 1949) and, subsequently, the number of chl molecules per reaction center (Babcock et al., 1983), we have found that illumination at 77 K for 10 min photooxidized 0.90 ± 0.10 molecules of cytochrome b_{559} per PSII (average of three measurements). It is apparent that cytochrome b_{559} was the only site photooxidized by 77 K illumination, since Figures 4a and 6b show that, aside from the Fe(II)–Q_A^- EPR signal, no other light-induced EPR signals were observed. This result is consistent with the quantitation of the cytochrome b_{559} EPR signal, which gives essentially one molecule of photooxidzed cytochrome b_{559} per PSII, as expected in PSII samples containing DCMU.

Figure 3 shows the dependence on illumination temperature of the amplitudes of the EPR signals displayed in Figure 1. It is seen that one stable charge separation occurred throughout the temperature range of 77–220 K, since the yield of the Fe(II)–Q_A^- EPR signal remained constant. As the illumination temperature was increased from 77 to 130 K, the g = 4.1 EPR signal was produced at the expense of the cytochrome b_{559} EPR signal. Increasing the temperature of illumination further to 220 K caused the production of the multiline S_2-state EPR signal at the expense of both the cytochrome b_{559} and the g = 4.1 EPR signals.

In order to determine whether cytochrome b_{559} and the g = 4.1 EPR signal species act as precursors to the S_2 state or as separate electron donors to $P680^+$, we warmed the illuminated samples for 2 min at 200 K in the dark. As can be seen in Figure 4, the cytochrome b_{559} and the Fe(II)–Q_A^- EPR signals were not affected, whereas the g = 4.1 EPR signal yielded the multiline EPR signal after dark incubation at 200 K. Close examination of the data also reveals an inverse relationship between the photooxidized cytochrome b_{559} EPR signal intensity and the multiline EPR signal obtained after annealing at 200 K in the dark. Figure 5 shows that, indeed, as the illumination temperature was increased from 77 to 220 K, the decrease in intensity of the cytochrome b_{559} EPR signal was directly paralleled by an increase in the multiline EPR signal intensity after annealing. Furthermore, the curves intersect at about 135 K, where roughly 50% of the maximum intensity of each EPR signal was produced. These results show that the g = 4.1 EPR signal is a precursor of the S_2 state, as suggested by Casey & Sauer (1984), and that cytochrome b_{559} and the S_1 state compete for electron donation to $P680^+$. The rate constants for the different pathways must be sufficiently temperature dependent, so that at 77 K $P680^+$ is reduced almost exclusively by cytochrome b_{559}, whereas at 200 K the S_1 state is the electron donor.

Cytochrome b_{559}. We have also investigated the electron-transfer steps that occurred when electron donation from cytochrome b_{559} was prevented. In order to accomplish this, we chemically oxidized cytochrome b_{559} with 2 mM K_2IrCl_6. Quantitation of the cytochrome b_{559} EPR signal produced by chemical oxidation showed that 2.1 ± 0.2 molecules of cytochrome b_{559} were present per PSII (average of five measurements). In order to determine whether both cytochrome b_{559} molecules are photochemically active, we have illuminated an untreated, dark-adapted PSII sample at 77 K for 10 min, as described in the previous section, then warmed it in darkness at 240 K for 2 min in order to allow Q_A^- to transfer an electron to Q_B, and then illuminated it again at 77 K for 10 min. As a result of the second illumination, the intensity of the g = 3.0 turning point of the EPR spectrum of oxidized cytochrome b_{559} increased by 50%. Thus, both molecules of cytochrome b_{559} present in PSII preparations can participate in low-temperature photochemistry of PSII.

Chemical oxidation of cytochrome b_{559} did not affect the ability to photooxidize the g = 4.1 and multiline EPR signal species at 130 and 200 K, respectively. Thus, the illumination temperature profiles presented in Figure 3 at temperatures higher than 130 K do not depend on the oxidation state of cytochrome b_{559}. Moreover, the line shapes of the g = 4.1 and multiline EPR signals were unaltered when cytochrome b_{559} was chemically oxidized. This result rules out the close association of cytochrome b_{559} with the g = 4.1 and multiline EPR signal species. Figure 6d shows, however, that a 10 G wide EPR signal centered at g = 2.0024 ± 0.0003 was produced by illumination at 77 K of samples in which cytochrome b_{559} was chemically oxidized. Quantitation shows that this EPR signal accounts for 95% of the reaction center concentration. A similar EPR signal, present in $K_3Fe(CN)_6$-treated thylakoid membrane samples, has been reported by Visser &

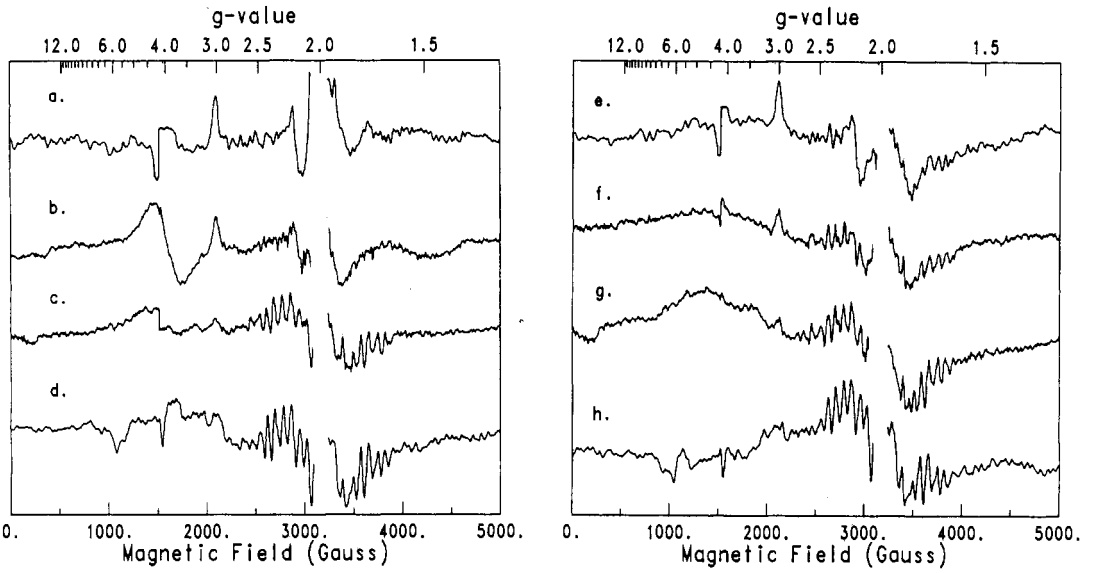


FIGURE 4: Effect of illumination temperature (a–d) and subsequent 2-min incubation at 200 K in the dark (e–h) on the EPR spectra of 4-h dark adapted PSII membranes treated with 50 μM DCMU: (a and e) 77 K illuminated minus dark; (b and f) 130 K illuminated minus dark; (c and g) 160 K illumination minus dark; (d and h) 200 K illuminated minus dark. Instrument conditions were as in Figure 1.

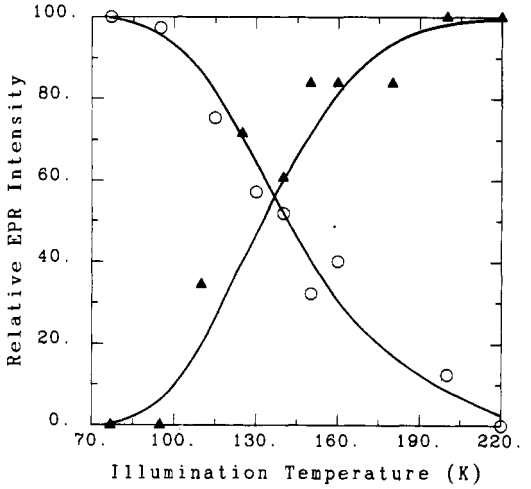


FIGURE 5: Effect of illumination temperature on the amplitudes of the photooxidized cytochrome b_{559} (circles) and the multiline EPR signals (triangles). The EPR signals were measured after 2 min of dark incubation at 200 K in order to allow for conversion of the g = 4.1 species to the multiline species. Data analysis was as in Figure 3. Instrument conditions were as in Figure 1.

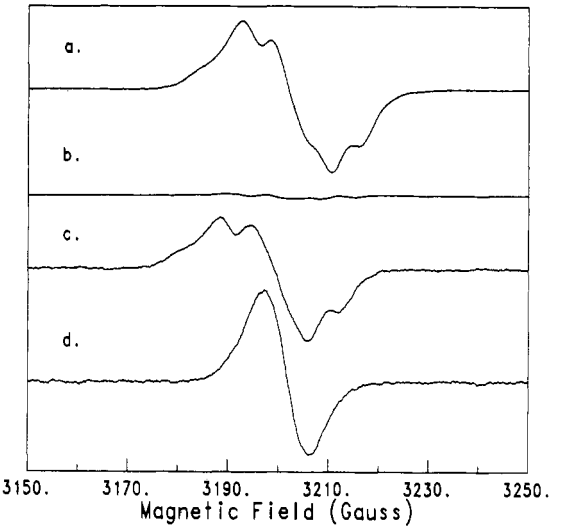


FIGURE 6: Effect of 77 K illumination on the g = 2.0 region of the EPR spectrum of PSII membranes: (a) 4-h dark-adapted PSII membranes; (b) 77 K illuminated minus dark-adapted PSII membranes; (c) 4-h dark-adapted PSII membranes treated with 2 mM K_2IrCl_6; (d) 77 K illuminated minus dark-adapted, K_2IrCl_6-treated PSII membranes. Instrument conditions were as in Figure 1, except that the modulation amplitude was 2 G and sample temperature was 15 (a and b) or 100 K (c and d).

Rijgersberg (1975), who tentatively assigned it to a chlorophyll cation radical. On the other hand, carotenoid cation radicals are also known to be photochemically generated in PSII preparations that have been treated with phenolic herbicides (Mathis & Rutherford, 1984) and the so-called ADRY reagents (Velthuys, 1981; Schenck et al., 1982). The g value of 2.0024 for the 10 G wide EPR signal that we observe after 77 K illumination of K_2IrCl_6-treated PSII membranes seems to be more consistent with that of a chlorophyll cation radical [Okamura et al. (1982) and references cited therein], however. Regardless of the identity of this radical EPR signal, our results indicate that it was an electron donor to $P680^+$ in freshly prepared PSII membrane samples, but only when other electron donation pathways were prevented.

It is also possible that an S_2-state precursor could be generated by 77 K illumination of samples where cytochrome b_{559} was chemically oxidized. In order to test this idea, we illuminated K_2IrCl_6-treated PSII membranes at 77 K for 20 min and then warmed the sample at 140 and 200 K in the dark. The amplitude of the 10 G wide EPR signal was not changed, and neither the g = 4.1 nor the multiline EPR signals could be produced in this manner, thus ruling out the existence of a precursor to the g = 4.1 or multiline EPR signal species that can be photooxidized at 77 K.

Two Electron Transfer Reactions in PSII. Brudvig et al. (1983) have shown that illumination of thylakoid membranes in the absence of DCMU at temperatures higher than 200 K resulted in a decrease in amplitude of the S_2-state EPR signal, since the $S_2 \rightarrow S_3$ transition became allowed. We performed experiments in the 190–235 K illumination temperature range in order to probe the intermediates that are generated during the $S_2 \rightarrow S_3$ transition.

Figure 7 shows that extensive illumination at 190 K did not induce the $S_2 \rightarrow S_3$ transition to a significant extent, since the amplitudes of the Fe(II)–Q_A^- and S_2-state multiline EPR signals decreased by only 20% from their maxima. In this experiment, no perceptible amount of the g = 4.1 EPR signal

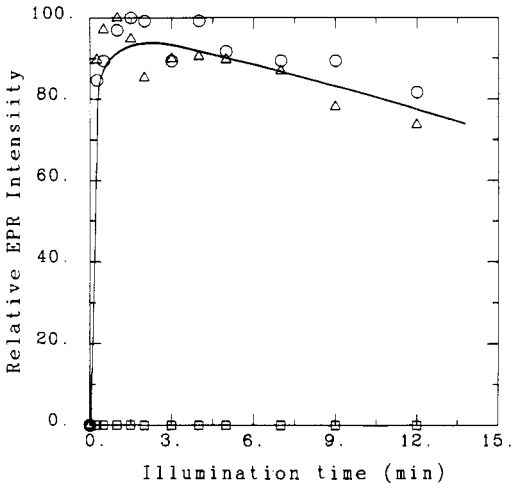


FIGURE 7: Illumination of 4-h dark-adapted, untreated PSII membranes at 190 K: multiline EPR signal (triangles); Fe(II)–Q_A^- EPR signal (circles); $g = 4.1$ EPR signal (squares). Instrument conditions were as in Figure 1; data analysis was as in Figure 3.

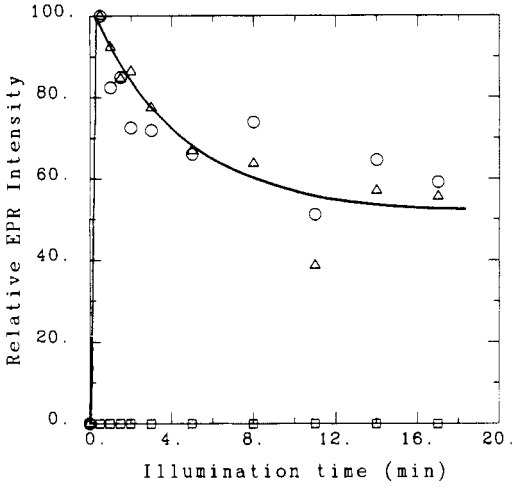


FIGURE 8: Illumination of 4-h dark-adapted, untreated PSII membranes at 225 K: multiline EPR signal (triangles); Fe(II)–Q_A^- EPR signal (circles); $g = 4.1$ EPR signal (squares). Instrument conditions were as in Figure 1; data analysis was as in Figure 3.

was detected throughout the 12 min of illumination.

The same experiment was performed at higher illumination temperatures, i.e., 225 K (Figure 8) and 235 K (Figure 9). At 225 K, the decrease in intensity of the S_2-state EPR signal was paralleled by a decrease in the Fe(II)–Q_A^- EPR signal amplitude, which clearly demonstrates that the $S_2 \rightarrow S_3$ transition was facilitated under these illumination conditions. At 235 K, the slower decrease of the Fe(II)–Q_A^- EPR signal with respect to the S_2-state EPR signal suggests either that a third turnover of the OEC may have occurred in some of the reaction centers at this temperature or that the Fe(II)–Q_A^- EPR signal has undergone a line-shape change. No contribution at $g = 4.1$ could be detected at any time during the illumination time course at 190, 225, or 235 K, however. This result suggests that the $g = 4.1$ EPR signal species is not an intermediate electron carrier between S_2 and S_3. Moreover, we did not observe any EPR signals that could be attributed to the S_3 state. Apparently, the Mn-containing site in the S_3 state is in an even-spin or perhaps diamagnetic state that is not observable by EPR spectroscopy at X-band.

Discussion

Our investigation of the one electron transfer events in PSII in the temperature range of 77–220 K suggests that, during

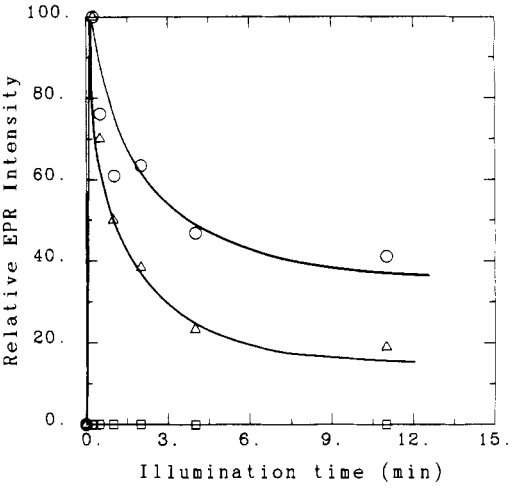
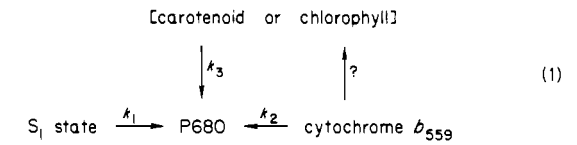


FIGURE 9: Illumination of 4-h dark-adapted, untreated PSII membranes at 235 K: multiline EPR signal (triangles); Fe(II)–Q_A^- EPR signal (circles); $g = 4.1$ EPR signal (squares). Instrument conditions were as in Figure 1; data analysis was as in Figure 3.

the irreversible charge separation produced by illumination, S-state advancement competed with alternate pathways of electron donation. These are summarized in eq 1.



(1)

Below 100 K, k_1 is small relative to k_2 or k_3, and a species other than S_1 is oxidized. According to our results, cytochrome b_{559} donates preferentially to $P680^+$ in this limit. Above 200 K, k_1 is large relative to k_2 or k_3, and hence, the S_1 state donates preferentially to $P680^+$ at higher temperatures. Furthermore, a third pathway is possible if cytochrome b_{559} is oxidized by chemical means. The identity of this alternate donor species, which exhibits a 10 G wide EPR signal centered at $g = 2.0024$, cannot be determined from these studies, but there is reason to believe that a chlorophyll radical (Visser & Rijgersberg, 1975) may be involved. It is also possible that a carotenoid may be the alternate donor to $P680^+$ in K_2IrCl_6-treated PSII samples (Nugent et al., 1982; Shenck et al., 1982; Mathis & Rutherford, 1984). Our results, however, do not rule out the possibility that cytochrome b_{559} is oxidized via the carotenoid or chlorophyll radical, as indicated in eq 1.

Previous reports on the roles of cytochrome b_{559} and carotenoids in PSII photochemistry have suggested that electron donation by these species may not have a physiological role, since neither species has been observed to act as an electron donor to $P680^+$ under conditions where O_2 evolution occurs. Both species, however, can act as electron donors under conditions in which O_2 evolution is inhibited, such as treatment with herbicides (Mathis & Rutherford, 1984) or ADRY reagents (Velthuys, 1981; Schenck et al., 1982). Our results support the conclusion that cytochrome b_{559} and the 10 G wide EPR signal species are not electron donors of physiological importance in PSII, since their photooxidation required the blockage of electron donation from the S_1 state. Our data also show that neither oxidized cytochrome b_{559} nor the 10 G wide EPR signal species are precursors of the S_2 state.

The electron-transfer scheme presented in eq 1 does not take into consideration the possibility that an intermediate electron carrier, usually termed Z (Bouges-Bouquet, 1980), exists

between the Mn site and $P680^+$. Kinetic evidence (Boska & Sauer, 1984) strongly supports the conclusion that EPR signal II_{vf} (very fast), which is thought to arise from the oxidized form of Z (Babcock & Sauer, 1975; Blankenship et al., 1975; Warden et al., 1976), mediates electron transfer between the Mn site and $P680^+$. Our results do not contradict this conclusion, but they do show that if the EPR signal II species mediates electron transport at cryogenic temperatures, then it is not stably photooxidized under the conditions we have used. In this same vein, our results also show that other EPR signal II species are not stably photooxidized at 77 K or higher temperatures. It has been reported that a form of EPR signal II, referred to as signal II_{lt} (low temperature), can be photooxidized by illumination at 5–15 K (Nugent et al., 1982).

We have been able to quantitatively account for electron donation in PSII below 100 K by cytochrome b_{559} and the 10 G wide EPR signal species. Our data also allow us to bring forth a few relevant points concerning the quantitation of the S_2-state EPR signal species. Hansson et al. (1984) have reported that the S_2-state multiline EPR signal accounted for only 5% of the total reaction center concentration. The drawbacks of their procedures were (i) the EPR signal was generated by slowly freezing the sample in liquid N_2 while illuminating, so that not all of the sites were trapped in the S_2 state, and (ii) the quantitation was based on a model for the S_2 state that may not be correct [see de Paula & Brudvig (1985)]. The data presented in this paper, however, suggest that the S_2-state multiline EPR signal species produced by 200–220 K illumination of 4-h dark-adapted PSII membranes treated with DCMU accounts for 100% of the reaction center concentration. This conclusion is borne out by the following facts: (i) at 77 K, one molecule of cytochrome b_{559} per PSII was photooxidized, which allows us to conclude that all of the sites underwent a single stable charge separation; (ii) the intensity of the Fe(II)–Q_A^- EPR signal did not deviate substantially from 100% throughout the illumination temperature range studied, which, together with (i), supports the conclusion that a single stable charge separation was complete throughout the temperature range of 77–220 K; (iii) long-term dark-adapted PSII membranes contain 100% S_1 (Hanssum et al., 1985); (iv) the S_2-state multiline EPR signal species was the only EPR-active paramagnet generated by illumination at 200 K, thus providing evidence that only the S_1 state donated an electron to $P680^+$; (v) the yield of photooxidized cytochrome b_{559}, which can be directly quantitated, decreased in direct parallel to the increase in the S_2-state multiline EPR signal as the illumination temperature was increased from 77 to 200 K (Figure 5). Therefore, the maximum fraction of S_2-state multiline EPR signal species that we detect per reaction center must be close to unity. This estimate of the concentration of the multiline EPR signal species further confirms its association with the S_2 state of the OEC and dispels any possibilities that this EPR signal arises from some kind of spectroscopic artifact or an alternate electron transfer path that may not be essential for the functioning of the OEC.

The quantitation of cytochrome b_{559} in PSII has also been the subject of controversy over the last few years. Several groups have reported on the spectrophotometric determination of the cytochrome b_{559} content of PSII membranes prepared with Triton X-100. Lam et al. (1983) and Murata et al. (1984) have found that two molecules of cytochrome b_{559} per PSII reaction center were present in their preparations. The proportion of high- to low-potential forms varied from 1:4 (Lam et al., 1983) to 1:1 (Murata et al., 1984). Sandusky et al. (1983), however, detected one molecule of cytochrome b_{559} per PSII reaction center, of which 50% were in the high-potential form and 50% in the low-potential form. We have used EPR spectroscopy to determine the stoichiometry of cytochrome b_{559} in PSII membrane samples that were treated with a powerful oxidant, K_2IrCl_6. Our results agree with those of Lam et al. (1983) and Murata et al. (1984), since we have determined that two cytochrome b_{559} molecules per PSII reaction center were chemically oxidized in our samples. We have not determined the ratio of high- to low-potential cytochrome b_{559}, although we observed that both molecules of cytochrome b_{559} are photochemically active at 77 K.

The experiments described in this paper also clarify some of the controversial points concerning the $g = 4.1$ EPR signal. Since the $g = 4.1$ EPR signal was optimally photoinduced at 130 K, some 70 K colder than the temperature threshold for production of the S_2-state multiline EPR signal, it is attractive to think of it as an EPR signal from a carrier between the S states and $P680^+$. The inability to observe the $g = 4.1$ EPR signal as an intermediate between S_2 and S_3, however, does not support this interpretation. We propose that the $g = 4.1$ and S_2-state multiline EPR signals are simply different spectroscopic manifestations of the same site, with different structural features. This view is supported by previous work (de Paula & Brudvig, 1985) in which it was shown that the line-shape and magnetic properties of the S_2-state multiline EPR signal were strongly dependent on the length of dark adaptation and, in short-term dark-adapted samples, on the temperature of illumination.

In summary, our results point to a rather simple picture for electron donation in PSII. Provided that the temperature is high enough for S-state advancement to occur (above 200 K for $S_1 \rightarrow S_2$), only two donor species need be involved. These are the Mn-containing active site, which acts as the storage site for oxidizing equivalents, and only one electron carrier, referred to as Z, which mediates electron transfer from the Mn site to $P680^+$. If S-state advancement is prevented by low temperature or inhibitors, then the powerful oxidant generated by charge separation in PSII leads to oxidation of nonphysiological electron donors such as cytochrome b_{559}, a carotenoid, or a chlorophyll molecule.

Registry No. $P680^+$, 58814-97-4; cytochrome b_{559}, 9044-61-5.

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