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Electron-Transfer Events near the Reaction Center in O_2-Evolving Photosystem II Preparations†

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Received February 23, 1988; Revised Manuscript Received April 28, 1988

ABSTRACT: Time-resolved ESR has been used to study electron-transfer reactions in oxygen-evolving photosystem II membrane fragments. The exogenous acceptor dichlorobenzoquinone (DCBQ) is reduced by photosystem II; the ESR spectrum of the resulting DCBQ radical overlaps the center but not the wings of the ESR spectra of the endogenous tyrosine radicals Y_D^+ and Y_Z^+. Here Y_Z^+ denotes the species that is involved in electron transfer between the reaction center chlorophyll, P_{680}, and the manganese-containing, oxygen-evolving complex, and Y_D^+ denotes the stable photosystem II radical. By using appropriate magnetic fields, we recorded kinetic transients of Y_Z^+ under repetitive flash conditions with DCBQ present. We also used 1 mM $K_3Fe(CN)_6$ as an exogenous acceptor when recording kinetic traces of Y_Z^+, although at concentrations above 5 mm we observe an additional signal that could be due to P_{680}^+. The kinetic traces of Y_Z^+ obtained with DCBQ or 1 mM $K_3Fe(CN)_6$ are similar and show two phases. The slower phase has a half-time of 1.2 ms and corresponds to the reduction of Y_Z^+ by the S_3 state; the faster phase reflects reduction of Y_Z^+ by both S_1 and S_2. By using flowing, dark-adapted PSII membranes, we resolved the $Y_Z^+S_1$ reaction ($t_{1/2}$ = 100 μs) on the first flash and found it to be significantly faster than the $Y_Z^+S_2$ reaction ($t_{1/2}$ = 300 μs) which occurs on the second flash. A high-resolution ESR spectrum of Y_Z^+ in O_2-evolving PSII membranes was obtained with gated integration techniques and found to be similar to the spectra of Y_D^+ and of Y_Z^+ in inhibited membranes. Thus, the magnetic interaction between spins on Y_Z^+ and the manganese in the oxygen-evolving complex broadens the Y_Z^+ spectrum negligibly. These results support the idea that a single electron carrier, Y_Z, operates between P_{680}^+ and the manganese ensemble in the oxygen-evolving complex and functions on all four S-state transitions.

In the photosystem II reaction center, oxidizing equivalents generated by a specialized chlorophyll, P_{680},[1] are transferred to a manganese-containing, oxygen-evolving site by way of an intermediate electron donor, which has been called Z or D1 [for reviews, see Babcock (1987) and Ghanotokis and Yocum (1985)]. This species was first observed by use of electron spin resonance to study thylakoids that had lost the ability to evolve oxygen through manganese depletion. In these preparations,

† This work was supported by the Photosynthesis Program of the USDA Competitive Research Grants Office and the McKnight Foundation.
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[1] Abbreviations: DCBQ, 2,5-dichlorobenzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ESR, electron spin resonance; OEC, oxygen-evolving complex; PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; P_{680}, primary donor of PSII; P_{700}, primary donor of PSI; Q_A, primary acceptor of PSII; Q_B, secondary acceptor of PSII; Tris, tris(hydroxymethyl)aminomethane; Y_D^+, stable tyrosine radical of PSII; Y_Z, electron donor of PSII, formerly called Z.

0006-2960/88/0427-5848$01.50/0

the Z^+ ESR signal is observed in the light and decays after a flash in about 100 ms (Babcock & Sauer, 1975). Recent work has indicated that the Z^+ species most likely corresponds to a tyrosine residue in the photosystem II core (Barry & Babcock, 1987, 1988; Debus et al., 1988), and for this reason, we designate it as Y_Z in its neutral reduced form and as Y_Z^+ in its oxidized state. In this notation, the stable free radical that occurs in photosystem II and gives rise to the signal IIs ESR spectrum is Y_D^+.

In oxygen-evolving material, the ESR signal of Y_Z^+ decays in 1 ms or less (Blankenship et al., 1975) as it is reduced by the oxygen-evolving complex (OEC). The rates of the Y_Z^+/OEC redox reactions are S-state dependent and decrease as the number of stored oxidizing equivalents increases (Babcock et al., 1976; Dekker et al., 1984; Renger & Weiss, 1986). A low-resolution, time-resolved ESR spectrum of Y_Z^+ in oxygen-competent thylakoids was presented among the original observations on the radical (Blankenship et al., 1975). This spectrum, however, was obscured by overlap with that of P_{700}^+, particularly because decay components of both occur in the same time range, which made it difficult to separate the contributions of the two radicals to the transient ESR signal. In the work reported here, we have used PSII membranes in which the P_{700} content has been minimized to measure the Y_Z^+ ESR spectrum under O_2-evolving conditions. A more accurate determination of this line shape is of interest because inhibition of the OEC has been reported to lower the redox potential of Y_Z (Yerkes et al., 1983) and to alter the optical difference spectrum of $Y_Z^+ - Y_Z$ (Hanssum et al., 1985). Inhibition also alters the microwave power saturation properties of Y_Z^+ (Warden et al., 1976). These results imply that the state of intactness of the OEC may influence the ESR spectrum of Y_Z^+.

PSII membranes as isolated contain only a small amount of the native electron acceptor, so for experiments involving signal averaging, addition of an electron acceptor is necessary. In O_2-evolving PSII membrane fragments, the choice of acceptor is considerably more complex than in unfractionated thylakoid membranes owing to the depletion of the plastoquinone pool and to the increased accessibility of the polypeptide ensemble involved in the water-splitting process to exogenous agents. As a result, a useful acceptor must operate at a potential high enough to accept electrons from Q_A^- or Q_B^- and ideally should not impose a rate limitation on the overall process. Deleterious interaction of the acceptor with the oxygen-evolving complex must be avoided. Finally, conditions in which oxidation of the acceptor-side Fe^{2+} by the added acceptor is minimized must be found. We examined two commonly used acceptors, dichlorobenzoquinone and ferricyanide, with these considerations in mind. Under certain experimental circumstances both acceptors allow observation of additional ESR signals in PSII preparations. We used these signals to investigate several electron-transfer events that occur in the photosystem II reaction center, and we identified conditions where these signals would not interfere severely with Y_Z^+ kinetic and spectral measurements in these O_2-evolving preparations. These precautions have allowed us to measure the kinetics of Y_Z^+ reduction in repetitive flashing light conditions and after dark adaptation of the membranes, and we compare the results to the kinetics of manganese oxidation that have been measured optically. We present the high-resolution, time-resolved ESR spectrum of Y_Z^+ in O_2-evolving membranes and consider the implications of the result in terms of the distance constraints it implies for components involved in O_2 evolution. A preliminary account of some of this work has appeared (Hoganson et al., 1987).

Materials and Methods

Photosystem II membranes were prepared from spinach (Ghanotakis et al., 1984a), but the Triton X-100 to chlorophyll ratio was increased by 40% to yield a product with less contamination from photosystem I. Oxygen evolution rates between 800 and 1000 μmol of O_2 (mg of Chl) h^{-1} were recorded with 300 μM 2,6-dichlorobenzoquinone as the electron acceptor. P_{700} is present at less than 5% of the concentration of Y_D^+. The membranes were used at a chlorophyll concentration of 2–2.5 mg of chl/mL in a buffer that contained 0.05 M MES (pH 6), 0.01 M NaCl, and 0.4 M sucrose (SMN).

ESR measurements were carried out at room temperature with a Bruker ER 200D spectrometer equipped with a Varian TM011 cavity and interfaced to a Nicolet 1180 microcomputer (Yerkes, 1981). All experiments used 100-kHz field modulation and lock-in detection. Samples were contained in a Scanlon flat cell and, where noted, were pumped continuously from a reservoir to the cell by a Gilson Minipuls 2 peristaltic pump. Under flow conditions, the sample typically received 12–15 flashes on each pass through the ESR cavity. Some experiments were performed with dark-adapted membranes. Only one or two flashes were given, after which a fresh aliquot was pumped into the flat cell. The 30-mL sample was recirculated at a rate slow enough to allow each aliquot to receive 10 min for dark adaptation between illuminations. Saturating, 17-μs xenon flashes from a laboratory-built flashing light apparatus excited the sample. The flash lump firing circuitry was controlled by a Metrabyte CTM-05 timing card in a Zenith Data Systems ZW-158-42 personal computer.

Spin quantitation was performed by double integration of the first-derivative ESR spectrum. The signal from Y_D^+ (signal II) in untreated, preilluminated PSII membranes with known chlorophyll concentration was used as a spin standard (Babcock et al., 1983). The effect of partial microwave saturation of the Y_D^+ signal was corrected for; Y_Z^+ saturates at higher power (Warden et al., 1976; Yocum & Babcock, 1981) and was not corrected. Kinetic decay traces were fit to a single or a double exponential decay function by a nonlinear optimization program of local origin.

The time-resolved ESR spectra of Y_Z^+ were recorded at room temperature with a Stanford Research Systems SR250 gated integrator and SR245 computer interface. The integrator was modified to allow extended integration periods. The ESR signal from the lock-in amplifier was sampled by the gated integrator for a period of 700 μs beginning 120 μs after each flash. Delaying the integrator period was done to discriminate against any fast absorbance changes due to P_{680}^+, although this does decrease the signal to noise ratio as Y_Z^+ decays also. Spectra were acquired by sweeping the magnetic field at a rate such that the product of the sweep rate and the time constant of the integrator was 0.3 G. Capacitive coupling between the ESR spectrometer and the gated integrator was used as this allows us to sample only the Y_Z^+ transient and not the dark spectrum of Y_D^+. A control scan on oxygen-evolving PSII membranes during which the lamp was not fired showed only a flat base line, confirming that this approach eliminates contributions from static radicals.

By using this method, we were able to acquire spectral data directly without having to integrate kinetic data. Such an integration would have been required for data acquired every few tenths of a gauss in order to obtain a spectrum of comparable resolution to that which can be obtained by the gated integrator. The integrator has the additional advantage that the signal to noise ratio is increased uniformly across the entire

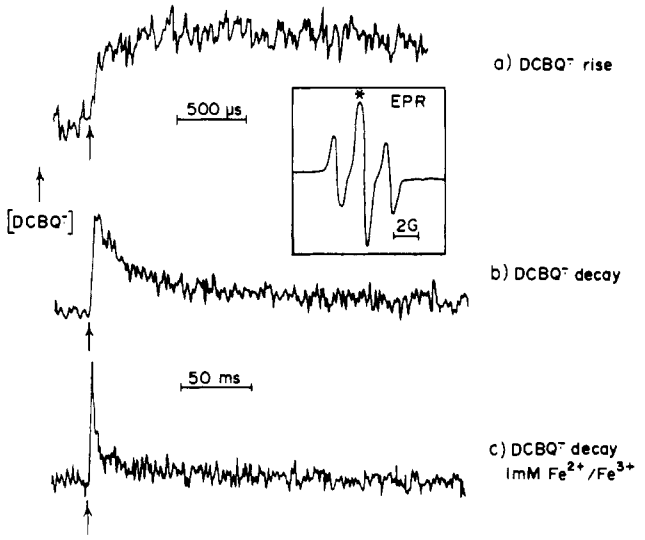

FIGURE 1: ESR detection of 2,5-dichlorobenzoquinone radical. Inset shows the dark-stable spectrum recorded at room temperature with 1.2-G field modulation. The sample contained 1 mM DCBQ, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and SMN (400 mM sucrose, 50 mM MES, pH 6.0, and 10 mM NaCl). The asterisk indicates the field used for kinetics experiments. (a) Formation kinetics of $DCBQ^{\cdot-}$ following flash illumination. The nonflowing sample contained PSII membranes (2.5 mg of Chl/mL), 0.5 mM 2,5-DCBQ, and SMN. Flash repetition rate was 1 Hz. 800 flashes were averaged; fresh samples were substituted after each 200 flashes. The rise is biphasic with half-times of 60 and 200 μs. (b) Decay kinetics of $DCBQ^{\cdot-}$. Conditions as in (a). 200 flashes were averaged. (c) Decay kinetics of $DCBQ^{\cdot-}$ with 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$.

spectrum for each additional scan of the spectrum, rather than at a single field value only, as would be the case if kinetic data were acquired. Using the gated integrator has the disadvantage that absorbance changes due to longer lived radicals, such as Y_Z^+ in damaged reaction centers, are not readily discriminated against. To monitor sample degradation during the course of the Y_Z^+ spectral measurements, we acquired kinetic traces with the magnetic field fixed at the low-field peak of the Y_D^+ spectrum. These showed that fewer than 10% of reaction centers were damaged, as judged by the amplitude of the signal that decayed in longer than 10 ms.

RESULTS

Exogenous Electron Acceptor Effects on Electron Transfer in O_2-Evolving PSII Preparations. 2,5-Dichlorobenzoquinone (DCBQ) is an effective acceptor and gives high rates of oxygen evolution with PSII membranes (Berthold et al., 1981). The ESR spectrum of the semiquinone of DCBQ (Figure 1 inset) consists of three lines in a 1:2:1 intensity pattern, with a g value of 2.0048 and a hyperfine coupling constant of 1.95 G. DCBQ differs from nonchlorinated benzoquinones in the stability of its semiquinone: at pH 6, the values of

$$K = [Q^{\cdot-}]^2/[Q][QH_2]$$

for DCBQ and for plastoquinone are 2×10^{-5} and 6.6×10^{-12}, respectively [calculated from data in Rich and Bendall (1980)]. Thus for concentrations of quinone and hydroquinone each at 0.5 mM, concentrations of the radicals are expected to be 2 μM and 1 nM for DCBQ and PQ, respectively. The former concentration of radical is readily observable by ESR. The spectrum shown in the inset in Figure 1 was measured with a solution of 1 mM DCBQ in the PSII membrane suspension buffer to which was added 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$. Double integration of the DCBQ signal and that of Y_D^+ in PSII membranes recorded under identical conditions allow us to estimate that the concentration of the $DCBQ^{\cdot-}$ radical in this solution is 1.7 μM. Similarly, the dark concentration of the DCBQ semiquinone in a solution of PSII membranes (2.5 mg of Chl/mL, in SMN) to which 1 mM DCBQ had been added was 3.9 μM. The higher concentration with the PSII membranes may be due to segregation of the quinone preferentially into the lipid phase. In a separate series of experiments, we found that 2,6-DCBQ has semiquinone stability and EPR spectral properties that are similar to those of 2,5-DCBQ.

Flash illumination of PSII membranes with DCBQ present yields a transient increase in the intensity of the ESR signal of $DCBQ^{\cdot-}$ (Figure 1). For PSII membranes at a reaction center concentration of 7.5 μM and DCBQ at 670 μM, we typically observed that the $DCBQ^{\cdot-}$ transient had a maximum amplitude corresponding to about 0.6 μM. Thus approximately 8% of the PSII reaction centers contribute to the maximum transient radical concentration. The transient rose biphasically with phases of about 60 and 300 μs, and it decayed in 25 ms (Figure 1). When ferricyanide and ferrocyanide are both present at 0.5 mM, the decay is accelerated to about 300 μs. The transient amplitude is approximately halved by the addition of 50 μM DCMU (data not shown.) No transient was observed when photosynthetic membranes were omitted from the sample.

When the membranes were solubilized by excess Triton X-100, a large $DCBQ^{\cdot-}$ transient was observed, which appeared initially as an absorbance decrease that was followed, in 50 μs, by an absorbance increase (data not shown). This indicates that the radical was formed in an emissively polarized state, presumably by reduction by triplet chlorophyll. Since the signal observed with intact membranes was not polarized, we believe it is related to electron transfer at the reaction center.

This transient increase in the $DCBQ^{\cdot-}$ concentration could be the result of either the reduction of the quinone by Q_A^- or the oxidation of the hydroquinone by Y_Z^+. The millisecond decay of the transient is probably due to disproportionation of the radical. Ferricyanide and ferrocyanide accelerate the decay by oxidation and reduction of the semiquinone, respectively. The fast rise of the signal suggests that DCBQ can accept electrons directly from Q_A^- with reasonable efficiency under flashing light conditions; reactions on the oxidizing side are likely to be slower than the observed 60-μs rise time due to the presence of the 17- and 23-kDa polypeptides and manganese which impede the diffusion of exogenous reductants to Y_Z^+ (Ghanotakis et al., 1984a,b). That a transient is observed suggests that $DCBQ^{\cdot-}$ need not bind strongly to the Q_B binding site, since the bound semiquinone of plastoquinone is not observable under room-temperature conditions. DCMU-insensitive DCBQ reduction suggests that reduction may occur without binding to the Q_B site. Of course, the majority of reaction centers, which may not contribute to the transient, might still bind the exogenous acceptor semiquinone reasonably tightly.

The ESR spectrum of $DCBQ^{\cdot-}$ is fairly narrow and allows one to observe signals beyond its wings. In particular, the low-field peak of the Y_D^+/Y_Z^+ spectrum is not obscured by the $DCBQ^{\cdot-}$ absorbance. Flash illumination, with detection at this low-field peak (g = 2.011), yields the rise and decay of the Y_Z^+ radical (Figure 2a). With 1 mM DCBQ as the added electron acceptor, the rise is limited by the applied instrument time constant (15 μs), and the decay occurs in two phases with decay half-times of 190 and 1140 μs (Table I). The latter of these decay times agrees well with the reported lifetimes

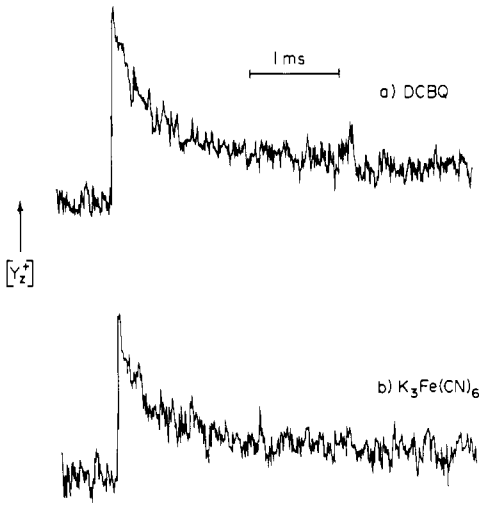

FIGURE 2: Y_Z^+ decay kinetics in a flowing, oxygen-evolving PSII membrane sample with [Chl] = 2.5 mg/mL. The instrument time constant was 15 μs. The added acceptors were (a) 1 mM 2,5-DCBQ and (b) 1 mM $K_3Fe(CN)_6$ and 1 mM $K_jFe(CN)_6$. 4.4-Gauss field modulation and 41-mW microwave power were used. 3200 flashes (a) or 2200 flashes (b) at 2 Hz were averaged. For each measurement a 10-mL sample was used. No off-resonance flash artifacts were subtracted for any of the kinetic traces shown in this paper.

Table I: Average Y_Z^+ Decay Parameters[a]

acceptor	$t_{1/2}$(fast) (μs)	amplitude (%)	$t_{1/2}$(slow) (μs)	amplitude (%)
2,6-DCBQ	175 ± 90	58 ± 15	1140 ± 400	42 ± 15
$K_3Fe(CN)_6$	195 ± 90	55 ± 15	1320 ± 530	45 ± 15

[a] Kinetic data were fit with a sum of two exponentials plus a constant. The tabulated values are the means and 95% confidence limits from six experiments with each acceptor.

of the $S_3Y_Z^+$ state, while the former decay time is somewhat faster than that reported for the $S_2Y_Z^+$ state (Babcock et al., 1976; Dekker et al., 1984; Ranger & Weiss, 1986). The peak amplitude of this signal corresponds to 0.43 spin per PSII. The slow phase contributes 42% of that, or 0.18 spin per PSII. If all centers were active and experienced no misses, one would expect this slow, ~1-ms decay phase to account for 0.25 spin per PSII under the steady-state conditions of this experiment. If misses average 15%, however, as in the experiments of Cole and Sauer (1987), then the fraction of centers showing Y_D^+ but not Y_Z^+ need by only 15% to account for the reduced amplitude of the 1-ms decay phase of the Y_Z^+ signal.

Ferricyanide is a useful acceptor as neither it nor its reduction product, ferrocyanide, has an ESR signal in the g = 2 region at room temperature. When the ferricyanide/ferrocyanide redox couple is present at 1 mM in each reagent, the Y_Z^+ radical can be observed to form and decay under repetitive flash illumination (Figure 2b). In these conditions, the decay traces of Y_Z^+ are similar in lifetimes and amplitudes to the decay traces observed when DCBQ is used as the exogenous acceptor (Table I). Decay phases of about 200 and about 1300 μs, with amplitudes of about 23% and 19% of the amplitude of the Y_D^+ signal, were observed. Because of the two exogenous acceptors yield similar Y_Z^+ kinetics, we conclude that neither acceptor under these conditions produces deleterious effects on the OEC. This argues against the possibility, mentioned above, that $DCBQH_2$ might be oxidized by the oxidizing side of photosystem II. Suspensions of PSII preparations show a different transient light-induced ESR signal when the ferricyanide concentration is greater than 3 mM. This signal has a spectrum suggestive of a chlorophyll radical (g = 2.0027, unresolved hyperfine); its narrowed line width

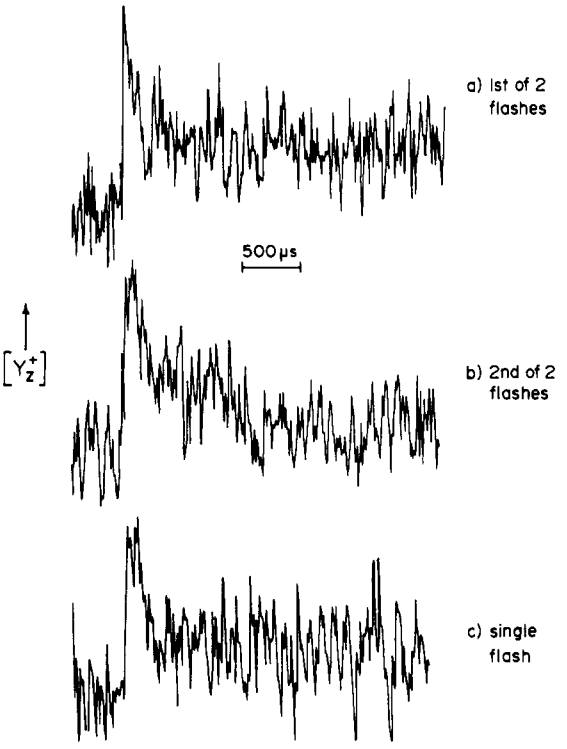

FIGURE 3: Y_Z^+ decay kinetics in a dark-adapted PSII membrane sample. (a and b) The first and second flashes, respectively, of a two-flash experiment; [Chl] = 2.0 mg/mL. Acceptors added were 0.25 mM 2,5-DCBQ, 0.25 mM $K_3Fe(CN)_6$, and 0.25 mM $K_4Fe(CN)_6$. The instrument time constant was 15 μs. 1571 pairs of flashes were averaged. 10 ms separated the two flashes. 4.4-G field modulation and 20-mW microwave power were used. (c) Result of a single-flash experiment with 0.25 mM DCBQ. 885 flashes were averaged.

(ΔH = 8.8 G) and 150-μs halflife suggest that it may be P_{680}^+. This radical and the effect of ferricyanide on photosystem II will be discussed in greater detail elsewhere (Hoganson & Babcock, 1988).

Y_Z^+ Kinetics in Dark-Adapted Membranes. To clarify the interpretation of the kinetics of the Y_Z^+ decay, particularly its role and decay time course in the $S_1 \rightarrow S_2$ transition, we performed some experiments on dark-adapted membranes. We chose to illuminate the sample with either one or two flashes. Since the stable S state in the dark in S_1, these flashes would advance the bulk of the centers to S_2 and S_3, respectively. Deactivation of S_2 and S_3 to S_1 occurs in the dark in times of approximately 1 and 3 min, respectively (Hanssum et al., 1985), and thus the 10-min dark period prior to flash excitation in our experiments allows for nearly complete dark adaptation. A third flash would not be useful in these experiments because the kinetics of the $Y_Z^+ + S_3 \rightarrow S_0 + Y_Z$ reaction are already resolved by the steady-state experiment (see above) and the decay time we observe for this transition agrees with earlier measurements (Babcock et al., 1976; Cole & Sauer, 1987). We have not been successful in monitoring the behavior of Y_Z^+ during the $S_0 \rightarrow S_1$ transition, which might be expected to dominate during a fourth flash, primarily because the decay of Y_Z^+ is likely to be too fast to be detected [30 μs (Dekker et al., 1984; Renger & Weiss, 1986)] and because the high miss factors (10–15%) associated with these optically dense samples lead to a considerable loss of S-state synchronization by the fourth flash. The latter complication is particularly bothersome and has also frustrated attempts to characterize the $S_0 \rightarrow S_1$ transition optically (Saygin & Witt, 1987). Furthermore, relaxation of S_0 to S_1 is very slow or negligible, so using more than two flashes complicates the

Table II: Y_D^+/Y_Z^+ Spectral Parameters

species	g value	line width (G)
Y_D^+/active	2.0044 ± 0.0002	21.4 ± 0.5
Y_Z^+/active	2.0040 ± 0.0006	21 ± 3
Y_Z^+/Tris	2.0043 ± 0.0005	21.4 ± 1.7

production of a single-valued initial S-state distribution. Giving only one or two flashes naturally produces a high fraction of centers in S_1 after dark adaption.

The result of an experiment in which two flashes spaced 10 ms apart were used is shown in Figure 3. 2,6-DCBQ, $K_3Fe(CN)_6$, and $K_4Fe(CN)_6$ were added as electron acceptors. The first flash produced a transient 52% as intense as the Y_D^+ signal and decaying with a half-time of 70–110 μs. The second flash produced a transient 56% as intense as the Y_D^+ signal but decaying with a half-time of 300–350 μs. A second experiment in which a single flash was used with DCBQ as the only added electron acceptor produced a transient 65% as great as the Y_D^+ signal and decaying in 90–120 μs (Figure 3c). Some experiments in which dark adaptation was incomplete gave transients 80% as great as the Y_D^+ signal and 200-μs kinetics on the first flash (not shown). The peak amplitudes of these fast signals are probably attenuated by the 15-μs instrument time constant. When the magnetic field was set to the zero-crossing point of the Y_D^+ spectrum, flashes given to a dark-adapted sample yielded a signal (not shown) decaying in milliseconds whose amplitude is readily accounted for by the small amount of P_{700}^+ that remains in these preparations. No submillisecond decay phase was observed at this magnetic field. These data indicate that Y_Z^+ functions as an intermediate in the oxidation of both S_1 and S_2 and that its reduction time depends on the S state of the OEC: $S_1Y_Z^+ \rightarrow S_2Y_Z$ has $t_{1/2} = 100$ μs, and $S_2Y_Z^+ \rightarrow S_3Y_Z$ has $t_{1/2} = 325$ μs. The amplitudes of the transient signals are consistent with the high oxygen-evolving activity of these preparations, suggesting that most centers are active. The similarity of the transients produced by a single flash or by the first of two flashes (spectra c and a of Figure 3, respectively) shows that ferricyanide, which was used as acceptor in the two-flash experiment, has not introduced complications related to the acceptor-side iron atom and that reduction of S_2 and S_3 has created the same S_1 state in both cases.

Time-Resolved Spectrum of Y_Z^+ in O_2-Evolving Samples. As described above, the choice of acceptor is an important consideration for the observation of the Y_Z^+ ESR spectrum, since some acceptor conditions can produce signals that overlap that of Y_Z^+. DCBQ has its own radical signal, and high concentrations of ferricyanide can inhibit electron transfer and lead to the observation of a radical that may be P_{680}^+. Ferricyanide and ferrocyanide at equal concentrations of 1 mM, however, do not have ESR signals of their own, nor do they induce spurious signals from the reaction center. Thus we have used this acceptor system for measuring the Y_Z^+ spectrum.

Using the gated integrator, we have obtained the spectrum of Y_Z^+ in both oxygen-evolving and Tris-washed PSII particles (Figure 4 and Table II). Also shown is the spectrum of Y_D^+, the stable PSII radical, obtained under the same conditions for comparison. All three spectra show a broad, asymmetric resonance. Except for uncertainties due to the experimental noise, the three spectra are very similar. The widths of the spectra are 21 ± 2 G. The *g* values for the three spectra are 2.0040, 2.0043, and 2.0044 for $Y_Z^+(O_2)$, Y_Z^+(Tris), and Y_D^+, respectively. All three spectra were recorded with a time constant large enough to distort slightly the zero-crossing region; in the oxygen-evolving case this was necessary in order to enhance the signal to noise ratio and was repeated for the

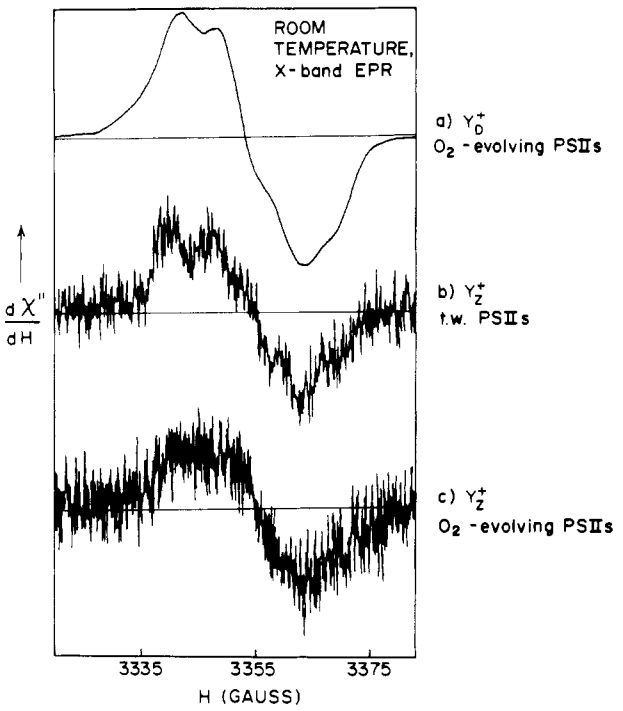

FIGURE 4: Time-resolved ESR spectra of Y_D^+ and Y_Z^+: (a) Y_D^+ in intact PSII membranes, (b) Y_Z^+ in Tris-washed membranes, and (c) Y_Z^+ in intact membranes. 1 mM $K_3Fe(CN)_6$ and 1 mM $K_4Fe(CN)_6$ were added as an acceptor system. The field modulation was 4.4 G, the microwave power was 20 mW, and the gated integrator was used. A gate 700 μs wide beginning 120 μs after the flash was used. The product of the effective time constant and sweep rate was 0.3 G. The spectra were scaled arbitrarily. For (b) and (c) flashes occurred at 2 Hz. (a) and (b) are single 1000-s scans. (c) is the average of 24 scans. 20-mL flowing samples were used and replaced after three scans.

Tris and Y_D^+ measurements for consistency. Thus the *g* value is slightly lower than the 2.0046 value that is recorded under nondistorting conditions. The slight lowering of the *g* value for the $Y_Z^+(O_2)$ spectrum, relative to the other two spectra, may be due to small contributions to the spectrum from P_{700}^+ or P_{680}^+, both of which have lower *g* values. They would contribute more to that spectrum because, under oxygen-evolving conditions, the Y_Z^+ amplitude after a flash is only about 50% that of Y_D^+, as discussed above, whereas after Tris washing the Y_Z^+ amplitude is the same as that of Y_D^+ (Babcock & Sauer, 1975).

One characteristic feature of the Y_D^+/Y_Z^+ line shape is the partially resolved hyperfine structure readily observed in two of the three spectra. The Y_Z^+ spectrum in the O_2-evolving sample does not have the requisite signal to noise ratio to make an absolute statement, but the overall shape of the spectrum, including the presence of three shoulders in appropriate locations, suggests that most, if not all, of the hyperfine structure is conserved between the three spectra.

Discussion

In order to study the kinetics of electron-transfer events in photosystem II particles, it is necessary to add an electron acceptor. The dichlorobenzoquinones as electron acceptors give high rates of steady-state oxygen evolution. They differ from other quinone species in the enhanced stability of their semiquinones. We have shown here that in a measurable fraction of the PSII reaction centers the semiquinone of DCBQ is not tightly bound to the Q_B binding site. This implies that this semiquinone is a fairly weak oxidant that may be inefficient in oxidizing the acceptor-side ferrous iron atom. Zimmerman and Rutherford (1986) found that the semi-

quinones of benzoquinone, dimethylbenzoquinone, and phenylbenzoquinone, formed by flash illumination at pH 6, oxidize this iron atom. The semiquinones derived from plastoquinone and dichlorobenzoquinone do not. Petrouleas and Diner (1987), however, found that $DCBQ^{\bullet-}$ does oxidize the iron atom at pH 7, although with only about a third the efficiency of the semiquinone of benzoquinone. Perhaps pH affects the ability of $DCBQ^{\bullet-}$ to oxidize the iron atom. Petrouleas and Diner also suggest that certain semiquinones, including that of DCBQ, may not bind tightly to the reaction center. Our results corroborate these conclusions with regard to DCBQ. This quinone has been used in optical measurements of photosystem II (Dekker et al., 1984), and whether the iron atom has been transiently oxidized is crucial to the interpretation of those data.

The hydrophilic electron acceptor, ferricyanide, also induces complications in photosystem II, particularly at high concentrations. At a concentration of 1 mM, however, it seems not to disturb Y_Z^+ reduction kinetics. We therefore used 1 mM ferricyanide/ferrocyanide as an exogenous electron acceptor while measuring the ESR spectrum of Y_Z^+ in intact PSII membranes. The spectrum of Y_Z^+ in O_2-evolving membrane fragments appears the same as that of Y_D^+, the stable radical of photosystem II. It is also the same as that of Y_Z^+ in inhibited membranes, regardless of whether the inhibition involves the release of manganese. The latter observation indicates that the procedure we used to obtain Y_Z^+ spin quantitation data in O_2-evolving samples (see above) is valid. Because our measurement, which averages the ESR signal between 120 and 820 μs after the flash, discriminates against shorter lived signals, our measured spectrum is weighted in favor of Y_Z^+ with the OEC in the S_3 state, although the $Y_Z^+S_2$ state does make an appreciable contribution to the spectrum. The comparable Y_Z^+ amplitudes we observe for the first and second flashes, in Figure 3, indicate that the Y_Z^+ spectrum is unlikely to be significantly perturbed in the S_1 state.

Our observation that the Y_Z^+ line shape is not altered by Tris washing indicates that the interaction of Y_Z^+ with manganese of the OEC in S_2 and S_3 is weak and does not strongly perturb the spin distribution on the Y_Z^+ radical. An altered spin distribution would produce a different hyperfine pattern, which is not observed. The manganese, however, does interact with Y_Z^+ strongly enough to alter its relaxation properties (Warden et al., 1976; Yocum & Babcock, 1981). It is interesting to compare the oxidizing side of PSII with the acceptor side of the bacterial reaction center where Q_A and Fe^{2+} are separated by the imidazole side chain of a histidine residue (Deisenhofer et al., 1985), yet the magnetic interaction is strong enough to broaden severely the Q_A^- resonance so that the signal is detectable only at very low temperature (Okamura et al., 1975). The interaction has considerable exchange character (Butler et al., 1984). If Y_Z^+ and Mn were this close, we expect that the Y_Z^+ resonance would be similarly broadened. There seems, however, to be less exchange interaction between Y_Z^+ and the OEC. This implies that the distances between Y_Z and the nearest manganese atom is fairly great (>10 Å) and that they may be separated by two or more amino acid residues.

Although there seems to be little exchange interaction, there is certainly a magnetic dipole–dipole interaction between manganese of the OEC and Y_Z^+ that increases the microwave power required to saturate Y_Z^+ (Warden et al., 1976; Yocum & Babcock, 1981). It would be interesting to determine whether the strength of he Y_Z^+/Mn spin–spin interaction depends on S state. Different oxidation states of manganese have different spin–lattice relaxation times and magnetic moments, and these would determine the ability of manganese to relax Y_Z^+. The ability of the OEC to relax Y_Z^+ does depend on the S state (Rutherford & Styring, 1987; Styring & Rutherford, 1988). Similar information for Y_Z^+ would be useful for understanding the structure of the manganase ensemble and might well suggest spatial and chemical heterogeneity in the ensemble. Such experiments are planned.

That a high-resolution spectrum of Y_Z^+ in both O_2-evolving and Tris-washed samples has the same line shape as that of Y_D^+ indicates that the two radicals have a similar orientation in the membrane. The same conclusion was reached by Berthold et al. (1981), who noted that the line shape of the radicals is sensitive to the orientation of the membrane in the ESR cavity and is different in thylakoids and PSII particles owing to different orientations of the membranes in these two preparations by the spectrometer magnetic field. That the Y_Z^+ line shape is independent of the presence of manganese, at least in S_2 and S_3, implies that its removal does not alter the orientation of Y_Z.

The similarity of the line shapes of Y_Z^+ and Y_D^+ under several conditions supports the model for the identity and location of these two radicals that has been developed recently. Y_D^+ has been shown to be a tyrosine free radical (Barry & Babcock, 1987, 1988), and its identification as Tyr^{160} on the D2 polypeptide has been reported (Debus et al., 1988). The high degree of homology between the D1 and D2 reaction center polypeptides (Trebst, 1986), particularly the essentially conserved sequence of nine amino acids that contains the Y_D^+ radical in a presumably transmembrane segment, strongly suggests that Y_Z^+ is the radical form of the corresponding Tyr^{161} located on the D1 polypeptide. The high degree of conservation at these particular sites and a presumed rotational symmetry between D1 and D2 similar to the C_2 symmetry that occurs for the L and M subunits of the bacterial reaction centers (Deisenhofer et al., 1985) together suggest that the protein secondary structure around the two radicals is very similar. The orientation of the phenol rings of Y_D and Y_Z in the membrane would then be virtually identical. Also similar between Y_D and Y_Z would be the angle between the plane of the phenol ring and the methylene hydrogens, which determines the hyperfine coupling of those nuclei to the electron spin (Barry & Babcock, 1988). Such a situation rationalizes well the similar membrane orientation dependencies of the ESR line shapes of Y_Z^+ and Y_D^+.

Knowing the ESR line shape of Y_Z^+ in oxygen-evolving membranes allows us to make use of the amplitudes as well as the decay times of the flash-induced Y_Z^+ ESR signals. Our kinetic results show clearly that Y_Z is transiently oxidized on three of the four S-tate transitions and that the reduction time of Y_Z^+ varies with S state. Furthermore, the amplitudes of the observed Y_Z^+ signals from dark-adapted samples, at 55–80% of the Y_D signal amplitude, approach one spin per PSII, providing quantitative support for the prevailing idea that Y_Z transfers electrons from the OEC to P_{680}. Previous ESR experiments had shown that Y_Z functions on the S_2 to S_3 and the S_3 to S_0 transitions (Babcock et al., 1976); our results confirm those observations and show that Y_Z also functions on the S_1 to S_2 transition. The transition from S_0 to S_1 probably occurs too fast for our instrument to detect reliably. Nevertheless, we conclude that Y_Z also functions in that transition. The reduction time of P_{680}^+ is 23 ns in both the $S_0 \rightarrow S_1$ and the $S_1 \rightarrow S_2$ transitions (Brettel et al., 1984). Since the rates are the same, it is likely that in both cases the same electron donor, Y_Z, reduces P_{680}^+. The reduction times of P_{680}^+ in the S_2 to S_3 and S_3 to S_0 transitions, however, are 3–10 times

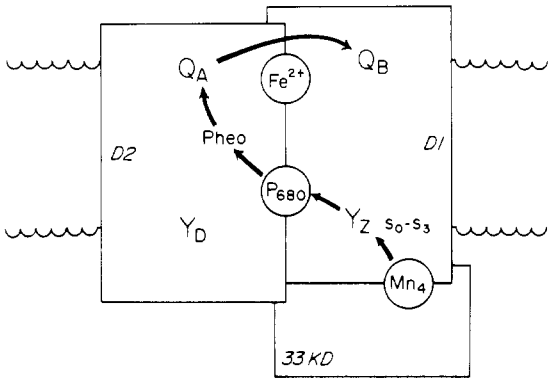

FIGURE 5: Electron transfer in photosystem II. Y is the biochemist's one-letter symbol for tyrosine; hence, Z and D are represented by Y_Z and Y_D, respectively.

longer than in the lower two S-state transitions. Prior to our measurements it was, therefore, conceivable that a donor other than Y_Z might reduce P_{680}^+ in the lower two S-state transitions. Our results now eliminate that possibility.

The amplitude of the Y_Z^+ signal in O_2-evolving preparations differs significantly between the experiments done with dark-adapted samples and those done with repetively flashed samples. In steady-state flashing light at a repetition rate of 2 Hz, the Y_Z^+ signal amplitude amounts to only 40–45% of the amplitude of the Y_D^+ signal, whereas in experiments with 10-min dark-adapted membranes, the Y_Z^+ signal amplitude approaches 80% of the Y_D^+ signal. This high yield of Y_Z^+ is consistent with the single flash yield (~90%) obtained with Tris-treated membranes and suggests that only a small fraction (~10%) of the centers in the untreated sample are not active in these experiments. The lower yield in the 2-Hz flashing light case is probably due to a rate limitation on the acceptor side.

It is known that formation of the S_2 multiline signal can proceed at temperatures as low as 160K (de Paula et al., 1985). Our finding that Y_Z^+ is involved in the S_1 to S_2 transition suggests that Y_Z^+ can be formed and can react normally at temperatures that low. This contradicts an earlier finding that Y_Z^+ was not observed at −40 °C (Warden et al., 1976). In those experiments, which used repetitive flashes, the low temperature may have prevented diffusion of electron acceptors to the Q_B site. This rate limitation would explain the failure to observe Y_Z^+ at −40 °C with repetitive flashes.

The reduction rates of Y_Z^+ reported here agree well with the rates of oxidation of manganese in the OEC that have been inferred from optical measurements (Dekker et al., 1984; Renger & Weiss, 1986). This agreement would seem to rule out the possibility that an additional electron carrier might function between Y_Z and manganese. Such a hypothetical component has been suggested in connection with previous time-resolved ESR results (Boska & Sauer, 1984; Boska et al., 1985; Cole & Sauer, 1987). The observations suggesting this were multiphasic decay kinetics and low amplitudes of the observed signal relative to the signal amplitude observed in inhibited preparations. Multiple decay phases may indicate multiple electron carriers, but they may also indicate charge accumulation in the OEC. The phases can be separated by S state by use of dark-adapted samples, as we do here. Diminished signal amplitudes may indicate electron flow through an alternate carrier, redox equilibration between the observed and an unobserved carrier, or slow removal of the electrons from the acceptor side. The last of these appears to be the case in our multiple flash experiments, but dark-adapted membranes are not subject to this rate limitation. Two flashes after dark adaptation both give large Y_Z^+ amplitudes.

The model for electron-transfer events in photosystem II supported by the data presented here is a simple linear one (Figure 5). P_{680} is photooxidized to P_{680}^+, a chlorophyll radical, which oxidizes a nearby tyrosine, Y_Z. Y_Z^+ in turn oxidizes manganese in the OEC. Leaving out heterogeneity in the manganese cluster, the scheme is linear with only Y_Z carrying electrons from manganese to P_{680}^+. The dependence of the electron-transfer rates on S state may be due to electrostatic effects resulting from varying charge distributions in the OEC in the different S states. Recent time-resolved optical measurements at 260 and 824 nm also support the idea that Y_Z is the only donor between P_{680} and the OEC (Gerken et al., 1987).

ACKNOWLEDGMENTS

We thank D. Ghanotakis, C. F. Yocum, B. Barry, R. Debus, J. de Paula, and G. Brudvig for helpful discussion and Y. Demetriou for preparing PSII membrane samples. D. H. Lillie wrote the program for analyzing decay kinetics.

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Bacteriorhodopsin Photoreaction: Identification of a Long-Lived Intermediate N (P, R_{350}) at High pH and Its M-like Photoproduct†

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Received February 25, 1988; Revised Manuscript Received April 6, 1988

ABSTRACT: An alkaline suspension of light-adapted purple membrane exposed to continuous light showed a large absorption depletion at 580 nm and a small increase around 350 nm. We attribute this absorption change to an efficient photoconversion of bR_{570} into a photoproduct N (P, R_{350}), which has a major absorption maximum between 550 and 560 nm but has lower absorbance than bR_{570}. N was barely detectable at low pH, low ionic strength, and physiological temperature. However, when the thermal relaxation of N to bR_{570} was inhibited by increasing pH, increasing ionic strength, and decreasing temperature, its relaxation time could be as long as 10 s at room temperature. N is also photoactive; when it is present in significant concentrations, e.g., accumulated by background light, the flash-induced absorption changes of purple membrane suspensions were affected. Double-excitation experiments showed an M-like photoproduct of N, ^{N}M, with an absorption maximum near 410 nm and a much longer lifetime than M_{412}. It may be in equilibrium with an L-like precursor ^{N}L. We suggest that N occurs after M_{412} in the photoreaction cycle and that its photoproduct ^{N}M decays into bR_{570}. Thus, at high pH and high light intensity, the overall photoreaction of bR may be approximated by the two-photon cycle $bR_{570} \rightsquigarrow M_{412} \rightarrow N \rightsquigarrow (^{N}L \leftrightarrow {}^{N}M) \rightarrow bR_{570}$, whereas at neutral pH and low light intensity it can be described by the one-photon cycle $bR_{570} \rightsquigarrow M_{412} \rightarrow N \rightarrow O_{640} \rightarrow bR_{570}$. The result of light-induced pH changes in purple membrane suspensions suggested that one proton is taken up from the medium during the thermal relaxation $N \rightarrow bR_{570}$ or the light reaction $N \rightsquigarrow bR_{570}$ (not during the reaction $M_{412} \rightarrow N$). At high pH and high ionic strength, a small amount of N appears to also be present in the dark, which implies that a thermal backreaction from bR_{570} to N also exists. The proposed modification of the photoreaction cycle model will require confirmation and possibly corrections by other techniques, e.g., vibrational spectroscopy. However, as it stands, it offers a satisfactory explanation of a variety of earlier observations which are inconsistent with the simple, generally used model $bR \rightsquigarrow K \rightarrow L \rightarrow M \rightarrow O \rightarrow bR$.

Bacteriorhodopsin (bR),[1] a transmembrane protein found in halobacteria, contains one molecule of retinal bound to the ε-amino group of a lysine residue via a protonated Schiff base linkage. Binding to the protein shifts the retinal absorption maximum >100 nm to the red and generates a broad absorption band near 570 nm. In the light, bR translocates protons across the cell membrane and generates an electric potential and a pH gradient which can be as large as 4 pH units (Stoeckenius et al., 1979; Kouyama et al., 1987). Preilluminated (light-adapted) bR contains only *all-trans-*

† Work in Japan supported by special coordination funds for promotion of science and technology, by a research grant from the Agency of Science and Technology of Japan entitled "Solar Energy-Photosynthesis", and, in part, by Grants-in-Aids from the Ministry of Education, Science, and Culture of Japan. Work in San Francisco supported by National Institutes of Health Grant GM-27057.
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[1] Abbreviations: bR, bacteriorhodopsin; pm, purple membrane; M^f, fast decay component; M^s, slow decay component.

0006-2960/88/0427-5855$01.50/0