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Redox Cofactor Interactions in Photosystem II: Electron Spin Resonance Spectrum of P_{680}^+ Is Broadened in the Presence of Y_Z^+†

Curtis W. Hoganson‡ and Gerald T. Babcock*
Department of Chemistry, Michigan State University, East Lansing, Michigan 48824
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ABSTRACT: The electron spin resonance spectrum of P_{680}^+ has been measured in photosystem II membranes at room temperature under repetitive flash excitation by using gated integration techniques. Oxygen evolution was inhibited in the samples used in these experiments, and the lifetime of the radical is extended into the 150–200-μs range. Three different treatments were used that allowed us to determine the spectral characteristics of P_{680}^+ when the paramagnetic species Y_Z^+ was also present. These results were compared to the P_{680}^+ spectral properties that we measured under conditions in which Y_Z was in its reduced, diamagnetic form. With Tris-inactivated membranes, where Y_Z^+ but not manganese was present, only a low P_{680}^+ signal amplitude could be measured, which precluded an accurate determination of the line width. With NaCl-washed membranes and membranes treated with $K_3Fe(CN)_6$, in which Y_Z^+ and manganese were both present during the measurement, the field-modulated P_{680}^+ spectrum is 8.9 G wide. This is 1 G wider than the spectrum measured when Y_Z remains reduced, as happens in membranes inhibited with NH_2OH. The broadening of the P_{680}^+ spectrum that occurs when its immediate donor is oxidized is attributed to a magnetic dipole–dipole interaction between P_{680}^+ and Y_Z^+. The extent of broadening allows us to estimate that the center-to-center distance between the two radicals is 10–15 Å.

Photosystem II uses light energy to create a chlorophyll cation radical, P_{680}^+,[1] which is a strong oxidant. A cluster of four manganese atoms in the oxygen-evolving complex (OEC) stores the equivalents generated by four consecutive photooxidations of P_{680} and catalyzes the oxidation of water. To convey oxidizing equivalents from P_{680}^+ to the manganese, a tyrosine residue (Barry & Babcock, 1987; Hoganson & Babcock, 1988; Gerken et al., 1988), called Y_Z, undergoes reversible one-electron oxidation to form a radical with a characteristic ESR line shape (signal II). Normally, these reactions are fast: the electron transfer from Y_Z to P_{680}^+ occurs in 20–250 ns; the electron transfer from the OEC to Y_Z^+ occurs in 30–1300 μs. Certain chemical treatments inhibit oxygen evolution and can greatly retard or entirely abolish these electron transfers, thereby lengthening the lifetime of the radicals and facilitating spectroscopic examination of them [for a review, see Babcock (1987)].

P_{680} and Y_Z are located in the reaction center of PSII, composed of the 32- and 34-kDa polypeptides, D1 and D2, respectively, and the 9- and 4-kDa polypeptides of cytochrome *b*-559 (Nanba & Satoh, 1987). P_{680} is probably bound to both D1 and D2 (Michel & Deisenhofer, 1986, 1988), and Y_Z is likely to be a residue of the D1 polypeptide (Debus et al., 1988a,b). Although detailed folding models for D1 and D2 are available (Trebst, 1986) and have received experimental support (Sayre et al., 1987), the spatial arrangement of the electron transfer components within the reaction center remains obscure.

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‡ Present address: Department of Biochemistry and Biophysics, Chalmers Institute of Technology, S-412 96 Goteborg, Sweden.

[1] Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ESR, electron spin resonance; OEC, oxygen-evolving complex; P_{680}, primary donor of PSII; PSII, photosystem II; Q_A, electron acceptor of PSII; Tris, tris(hydroxymethyl)aminomethane; Y_D^+, stable tyrosine radical in PSII; Y_Z, electron donor to P_{680}^+, also a tyrosine.

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In this paper we address the question of the organization of PSII by recording the spectrum of P_{680}^+ under two different conditions of PSII inhibition. In the first class of inhibitory treatments, electron transfer from manganese to Y_Z^+ has been prevented or slowed, and the measurement of the P_{680}^+ spectrum occurs with the tyrosine in its oxidized, free radical state. Under the second set of inhibitory treatments, electron transfer from Y_Z to P_{680}^+ is retarded, and recombination with Q_A^- determines the lifetime of the chlorophyll radical. The P_{680}^+ spectrum recorded under these conditions occurs with Y_Z in its reduced, diamagnetic state. Differences in the P_{680}^+ spectra for these two sets of conditions are attributed to interactions of the spin on P_{680}^+ with the spin on Y_Z^+; from these differences we estimate the distance between P_{680} and Y_Z.

Materials and Methods

Photosystem II membrane preparation from spinach and ESR measurements at room temperature were carried out as described previously (Hoganson & Babcock, 1988). The isolated membranes were suspended in 400 mM sucrose, 50 mM MES–NaOH (pH 6.0), and 15 mM NaCl (SMN). Tris inactivation was performed by incubating PSII membranes at a chlorophyll concentration of 0.5 mg/mL in 0.8 M tris(hydroxymethyl)aminomethane (pH 8.0) and 0.5 mM EDTA at 0 °C for 20 min under room light, followed by centrifugation and resuspension three times in SMN. Salt washing was as described by Ghanotakis et al. (1984b). Hydroxylamine treatment involved addition of 1.5 mM NH_3OHCl to intact PSII membranes followed by addition of from 2 to 10 mM $K_3Fe(CN)_6$ and illumination in the ESR flat cell for 10 s. Preillumination was omitted for the experiment shown in Figure 1. Xenon flashes of 17 μs excited the sample. Spin quantitation was performed by double integration of first-derivative ESR spectra. 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). Time-resolved spectra were recorded with a Stanford Research Systems SR250 gated integrator and SR245 computer interface, as described previously (Hoganson & Babcock, 1988). The integrator was modified to allow extended integration periods. The ESR signal after flashes was sampled by the gated integrator for a period of 150 or 250 μs. Computer control of the flashlamp and gated integrator via a CTM5 timing board (MetraByte Corp.) in a personal computer allowed us to sample the transient signal at different times on alternate flashes. This capability allowed subtraction of the longer lived signal of Y_Z^+ from the more rapidly decaying signal of P_{680}^+. In some experiments light intensity was varied by placing calibrated neutral density filters between the lamp and the ESR cavity.

Results

P_{680}^+ Line Shape in NH_2OH-Inhibited Preparations. In agreement with earlier ESR measurements (Ghanotakis & Babcock, 1983), we observe a strong flash-induced signal at the zero-crossing field of the Y_Z^+ spectrum upon addition of NH_2OH to PSII membranes. With short incubation times and without preillumination, the averaged signal resulting from 100 flashes is biphasic (Figure 1a). The fast phase decays with the time constant of our apparatus, about 20 μs, while the slow phase has a half-time of 200–300 μs. A two-flash experiment in which the flashes were separated by 1 ms is shown in Figure 1b. The second flash shows only the slower phase, suggesting that the fast phase reflects stable charge separation and that the slow phase reflects charge recombination without net reduction of an acceptor or net oxidation of a donor. After incubation for several minutes in the presence of 1.5 mM NH_2OH and more extensive illumination, the fast phase is replaced by the slower phase. The decay has a single-exponential halftime of 209 ± 17 μs (average of six determinations and 95% confidence limits). The flash-induced generation of this signal is not inhibited by the presence of DCMU (D. F. Ghanotakis, personal communication). Its spectrum, measured with the gated integrator, is shown in Figure 2a. It has a g value of 2.0022 and a width of 7.9 G (Table I). Double integration of the signal indicates that the radical is formed at a concentration of 1.0 spin per PSII. The signal shows no resolved hyperfine splittings and so is distinctly different from the spectrum of Y_Z^+, which is not detected after longer term incubation with NH_2OH [not shown, see Ghanotakis and Babcock (1983)], and of Y_D^+, the stable PSII tyrosine radical, which is observed and has its normal amplitude after illumination. The narrower line width of the transient radical also distinguishes it from an 11-G radical, sometimes observed in inhibited PSII preparations [e.g., de Paula et al. (1985)], that has been attributed to an accessory chlorophyll in the PSII reaction center (Thompson et al., 1988). On the basis of its narrow line width (Davis et al., 1979) and rapid decay, the transient signal that is produced by flashes is attributed to P_{680}^+ formed by photooxidation. The 200-μs decay of the signal corresponds to its reduction by the reduced acceptor, Q_A^-. The 20-μs phase may also be P_{680}^+ and may reflect its reduction by a donor other than Q_A^-, possibly Y_Z or NH_2OH. This faster reaction is clearly inhibited as incubation in NH_2OH proceeds. These observations confirm and extend earlier delayed light (Den Haan et al., 1976) and ESR measurements (Ghanotakis & Babcock, 1983) and are in good agreement with optical measurements (Van Best & Mathis, 1978; Ford & Evans, 1983; Weiss & Renger, 1986).

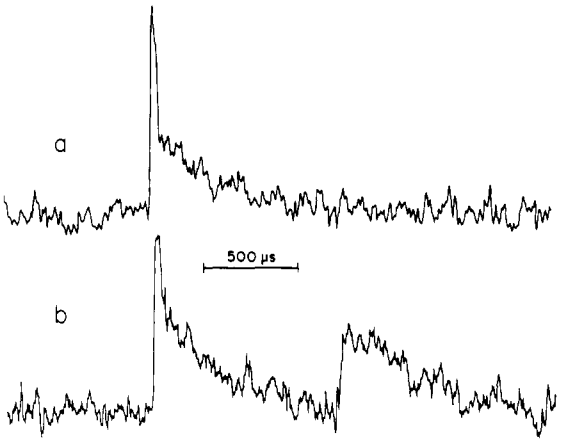

FIGURE 1: Kinetic decay traces of the ESR signal observed at the zero-crossing field of Y_D^+ in a suspension of PSII membranes with 1.5 mM NH_2OH and 3 mM $K_3Fe(CN)_6$ added. Ferricyanide has been added to maintain Q_A oxidized to allow charge separation to the $P_{680}^+Q_A^-$ state. 4.5-G field modulation and 20-mW microwave power were used. (a) Average signal from 100 flashes at 0.3 Hz; (b) average signal from 100 pairs of flashes at 0.3 Hz. 1 ms separated the two flashes in each pair.

Table I: P_{680}^+ Kinetic and Spectral Parameters[a]

treatment	half-life (μs)	g value	line width (G)
NH_2OH	209 (15)	2.0022 (3)	7.9 (3)
Tris	220 (30)	2.0025 (4)	8.5 (10)
$K_3Fe(CN)_6$			
2 Hz	153 (10)	2.0025 (3)	8.8 (5)
0.1 Hz	187 (30)		
NaCl, 2 Hz	157 (15)	2.0026 (3)	9.0 (7)

[a] Uncertainties in the last digit are given in parentheses.

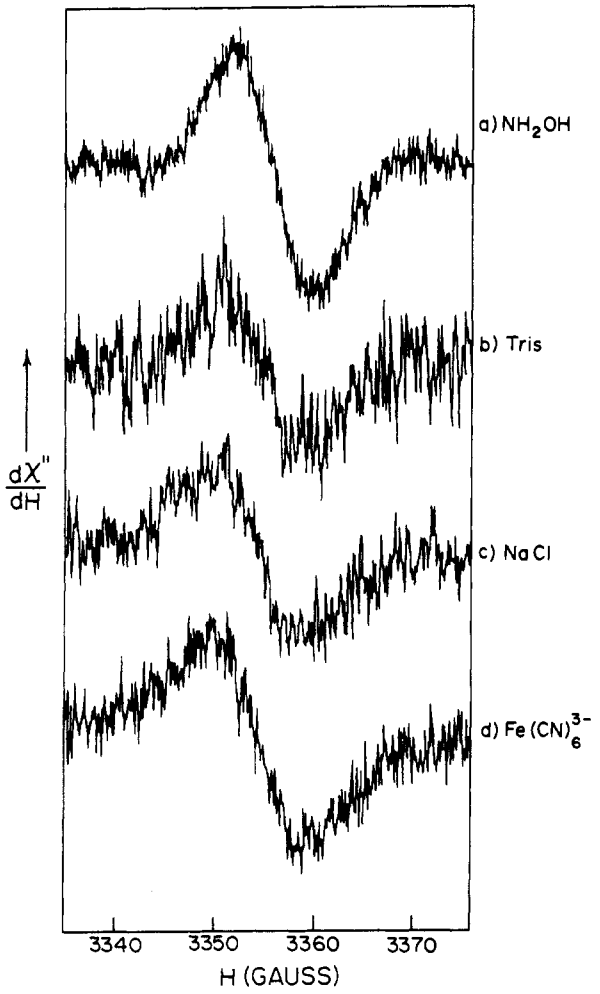

FIGURE 2: Spectra of P_{680}^+ in variously treated PSII preparations: (a) 1.5 mM NH_2OH and 3 mM $K_3Fe(CN)_6$; (b) Tris washed with 10 mM $K_3Fe(CN)_6$; (c) salt washed with 3 mM $K_3Fe(CN)_6$; (d) 10 mM $K_3Fe(CN)_6$. The number of scans averaged was (a) 3, (b) 20, (c) 10, and (d) 10. Flashes occurred at 2 Hz.

The mode of inhibition by NH_2OH is not known. It may involve a reversible oxime formation at one of the carbonyl groups of P_{680}, which might lower the redox potential of P_{680}^+ and prevent it from oxidizing Y_Z. Alternatively, a NH_2OH radical formed by photooxidation may be the inhibitory species.

P_{680}^+ Characteristics in Tris-Washed Preparations. Tris-washed PSII membranes, when the redox potential is poised so that Q_A is oxidized, form the Y_Z^+ radical during illumination. Flashes yield a Y_Z^+ concentration of nearly 1.0 spin per PSII. When 10 mM $K_3Fe(CN)_6$ and 10 mM $MgCl_2$ are present, these membranes develop an additional transient signal during exposure to 17-μs flashes of light. This signal is much weaker than that of P_{680}^+ observed with NH_2OH and corresponds to about 0.1 spin per PSII, but it has a similar lifetime and spectrum (half-time, 200 μs; g = 2.0025; width = 8.5 ± 1 G, Figure 2, Table I). To obtain the spectrum of P_{680}^+ in the presence of a large transient signal from Y_Z^+, P_{680}^+ was sampled for 150 μs beginning 50 μs after every second flash, while the signal of Y_Z^+ was sampled for the same period of time beginning 800 μs after alternate flashes and was subtracted electronically. The production of relatively stable P_{680}^+ in these experiments is attributed to double turnovers in a small fraction of PSII centers. With 17-μs flashes this is possible because two electron donors and two electron acceptors exist. The two donors are Y_Z and P_{680}^+. The two acceptors are Q_A, the physiological acceptor, and the acceptor side iron atom (Petrouleas & Diner, 1986), which can be oxidized to the ferric state by added ferricyanide. Electron transfer from Q_A^- to Fe^{3+} occurs in a few microseconds (Petrouleas & Diner, 1987), thereby enabling Q_A to accept a second electron from P_{680}^+ during a single flash. Electron donation from Y_Z to P_{680}^+ occurs in about 8–15 μs (Boska et al., 1983). Flashes produce the following sequence of events:

$$Y_Z P_{680} Q_A Fe^{3+} \xrightarrow{h\nu} Y_Z\, P_{680}^+\, Q_A^-\, Fe^{3+} \rightarrow$$
$$Y_Z^+\, P_{680}\, Q_A Fe^{2+} \xrightarrow{h\nu} Y_Z^+\, P_{680}^+\, Q_A^-\, Fe^{2+} \xrightarrow{\sim 200\ \mu s}$$
$$Y_Z^+\, P_{680}\, Q_A Fe^{2+}$$

The amplitude of the signal is small probably because double hits are required to produce P_{680}^+ and the duration of the light flash is not too different from the time required for the electron transfers on both the donor and acceptor sides. Flashes at pH = 7.5 gave a radical signal of the same intensity as at pH = 6.0. Attempts to increase the amplitude of the P_{680}^+ signal by double-flash techniques analogous to those in Figure 1 or by continuous background illumination were unsuccessful. It appears that the removal of electrons from Q_A^- constitutes a rate limitation that prevents a high yield of P_{680}^+ from being formed. Another possible explanation for the low amplitude of the P_{680}^+ signal is discussed below.

P_{680}^+ Line Width in Salt-Washed Preparations and after Ferricyanide Treatment. Salt-washed PSII membranes that have not been supplemented with Ca^{2+} show reduced oxygen evolution activity (Ghanotakis et al., 1984a). In these preparations, calcium depletion of the OEC occurs after a few turnovers of the OEC, and further electron transfers are inhibited (Dekker et al., 1984). In agreement with earlier work (Ghanotakis et al., 1984b), we observe that in continuous illumination slow reduction of Y_Z^+ occurs so that Y_Z^+ builds up to a steady-state concentration of about 0.6 spin per PSII. Under signal averaging conditions with flashes at 2 Hz, P_{680}^+ is produced and decays with a half-time of about 150 μs [see also Dekker et al. (1984)]. We have measured its ESR spectrum (Figure 2), again with electronic subtraction of the Y_Z^+ signal. The line width is 9.0 G, and the g value is 2.0026 (Table I). Double integration showed that 0.6 P_{680}^+ spin per PSII is formed with 2-Hz excitation.

When highly active oxygen-evolving PSII preparations are exposed to ferricyanide at concentrations greater than about 3 mM, a photoinduced ESR signal can be observed that is not observable when the ferricyanide concentration is 1 mM or less (Hoganson & Babcock, 1988). This signal is similar to that attributed above to P_{680}^+. When produced by repetitive flashing at 1 or 2 Hz, the radical signal has a single-exponential decay with a half-life of 153 ± 10 μs (average of five measurements and 95% confidence limits). The spectrum of the 153-μs signal was measured with the gated integrator by electronic subtraction of longer lived transients (Figure 2). The spectrum has a line width of 8.8 G and a g value of 2.0025 (Table I). Double integration indicates the radical is produced at a concentration of 0.6 spin per PSII. This is the same as the concentration of Y_Z^+ under these steady-state conditions. Thus, it is similar in all respects to the signal observed in NaCl-washed membranes. In these two preparations, the concentration of Y_Z^+ during the P_{680}^+ measurement is greater than 0.5 spin per PSII, and in both cases, the P_{680}^+ spectrum is approximately 1 G broader than in the preparation having Y_Z reduced. We conclude that the P_{680}^+ signal in these preparations is broadened by magnetic interaction with the spin on Y_Z^+.

A magnetic dipolar interaction between P_{680}^+ and Y_Z^+ is likely to cause enhanced spin relaxation of P_{680}^+ and to alter the microwave power saturation behavior of its ESR signal. We

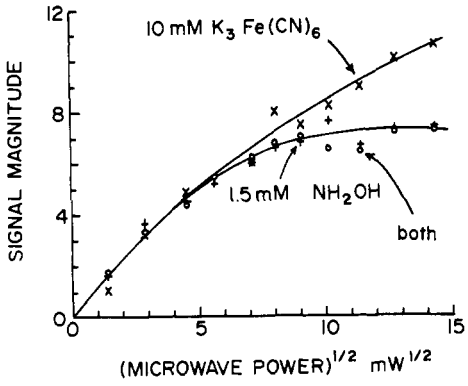

FIGURE 3: Power saturation of P_{680}^+: (×) 10 mM $K_3Fe(CN)_6$; (+) 1.5 mM NH_2OH and 3 mM $K_3Fe(CN)_6$; (○) 1.5 mM NH_2OH and 10 mM $K_3Fe(CN)_6$. 4.4-G field modulation was used. The transients from 200 to 400 flashes were averaged for each data point.

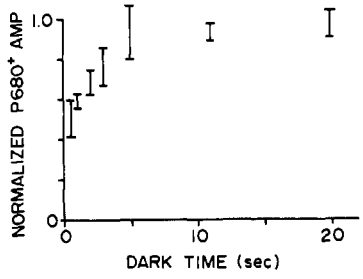

FIGURE 4: Flash repetition rate dependence of P_{680}^+ amplitude in PSII membranes with $K_3Fe(CN)_6$. 4.4-G field modulation and 20-mW microwave power were used. The transients from 200 flashes were averaged for each point.

also expect a reciprocal broadening of the Y_Z^+ spectrum by the spin on P_{680}^+. The latter effect has escaped detection thus far, most likely because it is obscured by the broader Y_Z^+ line shape and by the lower signal-to-noise ratio that results from the decreased signal amplitude (Hoganson & Babcock, 1988). We have, however, observed the predicted microwave power saturation effect. In Figure 3, we compared the power saturation of P_{680}^+ in membranes treated with either NH_2OH or $K_3Fe(CN)_6$. With NH_2OH present, P_{680}^+ is more readily saturated than when only 10 mM $K_3Fe(CN)_6$ is present. Addition of 10 mM $K_3Fe(CN)_6$ to NH_2OH-inhibited membranes did not alter the saturation behavior of P_{680}^+, so $K_3Fe(CN)_6$ cannot be responsible for the enhanced relaxation. These data indicate a magnetic interaction involving P_{680}^+ and support the idea that the broadening of the P_{680}^+ spectrum is due to a magnetic interaction with Y_Z^+. NH_2OH does release manganese from the OEC, so it is possible that manganese ions, which are retained in an EPR-silent state in the ferricyanide-treated membranes, affect the power saturation of P_{680}^+. The manganese cluster, however, is not sufficiently close to the Y_Z^+ species to broaden its spectrum (Hoganson & Babcock, 1988). Thus, we regard it as unlikely that the manganese cluster alters the magnetic resonance properties of P_{680}^+ directly; rather, any effects of the manganese ensemble are probably mediated by Y_Z^+.

The observation of P_{680}^+ in oxygen-evolving PSII preparations with 10 mM ferricyanide complicated our measurements of the Y_Z^+ radical (Hoganson & Babcock, 1988) and induced us to characterize further the phenomenon. The generation of the P_{680}^+ signal appears to require a double-turnover event similar to that described above for the generation of P_{680}^+ in Tris-inactivated membranes and that described by Jursinic (1981) for the first of a series of 3-μs flashes given to dark-adapted, oxygen-evolving chloroplasts in the presence of 1.5 mM $K_3Fe(CN)_6$. Two different experiments suggest this

Table II: Attenuation of Radical Signals with a 20% Transmitting Neutral Density Filter[a]

sample	species	repetition rate (Hz)	relative attenuated amplitudes (%)	number of quanta
Tris	Y_Z^+	1	64 (6)	1
Tris/NH_2OH	P_{680}^+	1	69 (10)	1
O_2/NH_2OH	P_{680}^+	1	65 (10)	1
O_2/$K_3Fe(CN)_6$	P_{680}^+	0.1	47 (3)	2
O_2/$K_3Fe(CN)_6$	Y_Z^+	0.1	44 (10)	2
O_2/$K_3Fe(CN)_6$	P_{680}^+	1	57 (9)	1–2

[a] Attenuated amplitudes are relative to the unattenuated amplitude. Uncertainties in the last digit are given in parentheses.

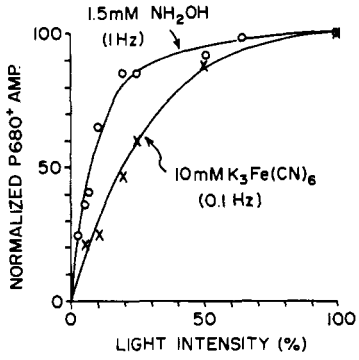

FIGURE 5: Light intensity dependence of P_{680}^+. Light intensity of the flashes was varied with neutral density filters. The samples contained either 1.5 mM NH_2OH and 3 mM $K_3Fe(CN)_6$ or 10 mM $K_3Fe(CN)_6$. 200–500 flashes were averaged for each data point. 4.4-G field modulation and 20-mW microwave power were used.

conclusion. The first is shown in Figure 4, which plots the amplitude of P_{680}^+ as a function of flash repetition rate. At rates greater than 0.2 Hz, the amplitude of P_{680}^+ decreases. Slow reoxidation of Q_A^- or the iron atom in the dark time between flashes could limit the yield of charge separation and of P_{680}^+. When 300 μM of the efficient acceptor 2,5-dichlorobenzoquinone was added to the preparation, the amplitude of the signal became independent of flash repetition rate up to 1.3 Hz (not shown). This observation indicates that the rate-limiting step occurs on the acceptor side of PSII, most likely the oxidation of the acceptor side iron. The data of Figure 4 suggest a pseudo-first-order rate of 0.36 s^{-1} for the limiting step, from which a second-order rate constant of 36 M^{-1} s^{-1} is calculated. Wraight (1985) found the iron atom (Q_{400}) to be oxidized by 0.25 mM $K_3Fe(CN)_6$ with a half-time of 2–3 min. From this, we calculate a second-order rate constant of 19 M^{-1} s^{-1}. The similarity between the latter rate constant and the rate constant we calculate supports the suggestion that oxidation of the acceptor-side Fe^{2+} contributes to the effect seen in Figure 4.

A double-turnover mechanism is also suggested by the flash saturation data of Figure 5, which shows the excitation intensity dependence of the P_{680}^+ signal in preparations containing either $K_3Fe(CN)_6$ or NH_2OH. Similar data on these and some other flash induced signals are also presented in Table II. The curves show that light more easily saturates the reaction in membranes inhibited by NH_2OH than in membranes treated with $K_3Fe(CN)_6$: half-saturation for the latter system occurs at ~2.4 times the light intensity required to half-saturate the former. Modeling this light intensity dependence behavior with one- and two-photon mechanisms (Ley et al., 1976) and a pigment bed size of 300 chlorophylls per reaction center revealed that 2.5-fold more light is required to half-saturate the double-hit mechanism, in good agreement with the data in Figure 5. Addition of 10 mM $K_3Fe(CN)_6$ to Tris-washed

PSII membranes or to PSII membranes with NH_2OH did not alter the light intensity dependencies of the Y_Z^+ or P_{680}^+ signals, respectively, in those membranes, indicating that $K_3Fe(CN)_6$ does not reduce the quantum yield of charge separation by disturbing excitation transfer to the reaction center. We conclude that after NH_2OH inhibition a single absorbed photon produces P_{680}^+. This is reasonable, because neither the manganese ensemble, which is disrupted by NH_2OH treatment, nor Y_Z is oxidized in the presence of NH_2OH. With $K_3Fe(CN)_6$ treatment, two photons are required to produce P_{680}^+, consistent with a double-turnover mechanism.

Discussion

Inhibition of oxygen evolution decreases electron transfer reaction rates on the donor side of photosystem II and extends the lifetime of the oxidized primary and secondary donors. In specific circumstances, such preparations can exhibit at one time both the primary and secondary donors in their one electron oxidized states. When both radicals are present in salt-washed or ferricyanide-inhibited preparations, they interact magnetically to produce a broadened P_{680}^+ spectrum. In contrast, addition of NH_2OH prevents oxidation of Y_Z by P_{680}^+, so a state $P_{680}^+ Y_Z$ is formed. The spectrum of P_{680}^+ in this case is approximately 1 G narrower than when Y_Z is oxidized. The spectrum of P_{680}^+ in Tris-washed membranes, which lack manganese, is not well resolved due to the low signal amplitude of P_{680}^+. This is unfortunate, because the presence of manganese in the OEC might also have an effect on the P_{680}^+ line width. Nevertheless, we believe that the broadening in the P_{680}^+ spectrum is due to Y_Z^+ because it is probably much closer to P_{680}^+ than is manganese.

Two different arguments indicate that the radical we observe is indeed P_{680}^+. The first is the narrower line width of the transient species relative to other radicals that have been reported in PSII. In particular, the 8–9-G signal we observe is significantly narrower than the 10–11-G radical that is observed under various conditions in PSII (de Paula et al., 1985; Rodriguez et al., 1988). A line narrowing, relative to monomer chlorophyll, is typical of reaction center cation radicals and has been discussed for P_{680}^+ (Davis et al., 1979). The second is the similarity in decay kinetics between our measurements and optical measurements of thylakoid or PSII membranes at 680 nm, where P_{680} absorbs, and at 820 nm, where P_{680}^+ absorbs. There have been numerous reports of 100–200-μs kinetics in optical studies of thylakoids inhibited by low pH or Tris (Mathis et al., 1976; Haveman & Mathis, 1976; Conjeaud et al., 1979; Conjeaud & Mathis, 1980). Our observation of two decay phases, with lifetimes of about 20 and 200 μs, after inhibition with NH_2OH agrees well with kinetic phases in optical measurements on thylakoids (Van Best & Mathis, 1978) and on PSII membranes (Ford & Evans, 1983; Weiss & Renger, 1986) that were attributed to P_{680}^+. Dekker et al. (1984) observed 200-μs kinetics in both optical and ESR experiments on NaCl-washed PSII membranes. Bock et al. (1988) used ESR and visible absorption to study radicals in PSII membranes inhibited by 600 mM acetate. Illumination with flashes at 10 Hz oxidized Y_Z entirely. They observed optical and ESR transient signals with decay phases with half-times of 530 and 170 μs whose ESR line widths were 14 and 8 G, respectively, that were attributed to P_{680}^+. The faster of these components may have the same origin as the signals we have observed in ferricyanide and salt-washed membranes. Decay phases of 600 μs have also been observed at 820 nm and were attributed to P_{680}^+ (Golbeck & Warden, 1985; Ford & Evans, 1985). We have not observed any signal corresponding to the 530–600-μs phases, which we suspect may be related to photooxidative damage of the reaction center caused by excitation at high repetition rates. Recent optical measurements on Tris-washed membranes have disclosed 160-μs decay kinetics at both 680 and 830 nm (Akabori et al., 1988). The correspondence of these optical decay phases having 150–200-μs kinetics with our ESR results supports our identification of the observed radical as P_{680}^+.

A theory to estimate the effect of dipolar interaction between randomly oriented pairs of unlike spins in a rigid lattice has been developed by Leigh (1970). This theory, which assumes that exchange interactions between the radicals are negligible, predicts quantitatively the broadening in the electron spin resonance line shape. The broadening depends on an interaction parameter

$$C = \frac{2\pi g\beta\mu^2 T_1}{r^6 h}$$

where g is the g value, β is the Bohr magneton, μ is the magnetic moment of the broadened spin, T_1 is the spin–lattice relaxation time of the spin causing the broadening, r is the distance between the two spins, and h is Planck's constant. Leigh's published results are for an isotropic Lorentzian line without hyperfine coupling. This theory presumes that the spins are fixed in space at a constant distance but randomly oriented. The reciprocal of the Larmour frequency should be less than the spin–lattice relaxation time of the spin causing the broadening, which should also be less than the spin–spin relaxation of the spin whose spectrum is broadened. Since the dipolar interaction depends on the angle, θ, between the laboratory field, $\mathbf{H}_0$, and the vector connecting the two spins, these conditions imply that as the dipolar interaction is increased the observed field-modulated ESR spectrum becomes progressively more a reflection of pairs of spins for which θ is near the magic angle (54.7°). For the radicals in PSII, the requirement of fixed distance and orientation is fulfilled. In our experiments, the reciprocal of the Larmour frequency is 10^{-10} s. For organic radicals at room temperature, T_2 is 10^{-6}–10^{-8} s. We take 10^{-6} s to be an appropriate estimate for the T_2 of P_{680}^+. The exact value does not enter into the calculation. The power saturation behavior of Y_Z^+ indicates that it has a somewhat longer T_1 than does aqueous Mn^{2+} which has a T_1 of about 10^{-9} s. A figure of 10^{-9}–10^{-8} s is a reasonable estimate for this T_1. In Leigh's theory, the estimated value of the distance has a one-sixth power dependence on T_1, so uncertainty in the value chose for T_1 still allows a meaningful estimate of the distance. These estimates for T_1 and T_2 satisfy the presuppositions of the theory. Presuming exchange between Y_Z^+ and P_{680}^+ to be negligible, we estimate that a center–center distance of 8–12 Å separates the two radicals.

Because the spectrum of P_{680}^+ is broadened by proton hyperfine interactions, Leigh's theory may not be applicable. Thus, we have used a second method to estimate the separation distance by evaluating the effect Y_Z^+ has on the local field at P_{680}^+. The magnitude of this dipolar field (H_z) in the direction of the laboratory field ($\mathbf{H}_0$) depends on the angle, θ, between $\mathbf{H}_0$ and the vector connecting the two radicals:

$$H_z = \mu(3\cos^2\theta - 1)/r^3$$

The membranes, and consequently Y_Z^+ and P_{680}^+, are immobilized and randomly oriented, so there is a distribution of angles and of H_z. The line shape of the unbroadened P_{680}^+ spectrum is Gaussian, presumably from unresolved proton hyperfine interactions. For a Gaussian distribution, the root mean square average of the variations from the center of the distribution is just half the peak to peak derivative line width.

The squared deviations from the mean that arise from various independent sources should be additive. Thus, the root mean square contribution of the dipolar field from Y_Z^+ can be readily calculated from the broadened and unbroadened P_{680}^+ line widths:

$$(8.9\ \text{G}/2)^2 = (7.9\ \text{G}/2)^2 + H_{z,\text{rms}}^2$$

$$H_{z,\text{rms}} = 2.05\ \text{G}$$

The root mean square of the z component of the dipolar field can be calculated by averaging the absolute value of H_z over the possible angles

$$H_z = 0.7698\mu/r^3$$

from which we calculate a center to center distance, r, of 15 Å. Because the assumptions used in this calculation seem more appropriate to the present case, we believe this method provides a better estimate of the distance between P_{680}^+ and Y_Z^+ than does Leigh's method.

The dipolar interaction between Y_Z^+ and P_{680} provides an alternate explanation to the one given above for the low amplitude of the P_{680}^+ signal in Tris-washed PSII membranes. Leigh (1970) suggests that the strength of the interaction between two spins should depend on the spin–lattice relaxation time of the spin causing the broadening, in this case Y_Z^+. Tris-washed membranes are depleted of manganese, so the relaxation time of Y_Z^+ is longer than that in membranes in which manganese relaxes Y_Z^+ (Warden et al., 1976; Yocum & Babcock, 1981). Thus, the dipolar interaction will have a larger effect in the Tris-washed membranes and would lead to a signal that is of lower amplitude but only slightly broadened.

This estimate of the distance between P_{680} and Y_Z (15 Å) is consistent with the likely positions of these two cofactors in the PSII reaction center. Debus et al. (1988a,b) have identified the tyrosine residue of the D2 polypeptide responsible for the Y_D^+ ESR signal and have suggested a probable site for Y_Z on the D1 polypeptide. Folding schemes for D1 and D2 (Trebst, 1986) locate these tyrosines in membrane spanning regions, consistent with recent dysprosium-induced relaxation studies on PSII preparations that indicate that these residues are buried fairly deeply in the PSII core (Innes & Brudvig, 1988). Deisenhofer et al. (1985), noting the amino acid sequence similarities of the PSII core polypeptides with the bacterial reaction center subunits, have suggested a probable location for P_{680} bound to histidines from D1 and D2. Both residues are within the membrane-spanning region of the reaction center near the lumenal side in helices that are adjacent to the C helix thought to carry the Y_z tyrosine.

Witt et al. (1986) estimated the distance between P_{680} and Y_Z by a different means. They assumed that the dependence of the electron transfer rate from Y_Z to P_{680}^+ on the S state of the OEC reflects the presence of an excess charge in the OEC in states S_2 and S_3. The method does not give a unique distance unless the distance between the manganese cluster and Y_Z or P_{680} is known. They found the electrostatic energy involved to be consistent with a Y_Z to P_{680} distance of 11 Å, assuming a distance of 19 Å between Y_Z and the OEC. This is in reasonable agreement with our estimate of the distance between Y_Z and P_{680}.

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Registry No. P_{680}^+, 58814-97-4; Y_Z^+, 96946-98-4.

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Chemical Probes of the Conformation of DNA Modified by *cis*-Diamminedichloroplatinum(II)†

Laurent Marrot and Marc Leng*
Centre de Biophysique Moléculaire, 1A Avenue de la Recherche Scientifique, 45071 Orléans Cedex 2, France
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ABSTRACT: The purpose of this work was to analyze at the nucleotide level the distortions induced by the binding of *cis*-diamminedichloroplatinum(II) (*cis*-DDP) to DNA by means of chemical probes. In order to test the chemical probes, experiments were first carried out on two platinated oligonucleotides. It has been verified by circular dichroism and gel electrophoresis that the binding of *cis*-DDP to an AG or to a GTG site within a double-stranded oligonucleotide distorts the double helix. The anomalously slow electrophoretic mobility of the multimers of the platinated and ligated oligomers strongly suggests that the platinated oligonucleotides are bent. The reactivity of the oligonucleotide platinated at the GTG site with chloroacetaldehyde, diethyl pyrocarbonate, and osmium tetraoxide, respectively, suggests a local denaturation of the double helix. The 5'G residue and the T residue within the adduct are no longer paired, while the 3'G residue is paired. The double helix is more distorted (but not denatured) at the 5' side of the adduct than at the 3' side. In the case of the oligonucleotide platinated at the AG site, the double helix is also more distorted at the 5' side of the adduct than at the 3' side. The G residue within the adduct is paired. The reactivities of the chemical probes with six platinated DNA restriction fragments show that even at a relatively high level of platination only a few base pairs are unpaired but the double helix is largely distorted. No local denaturation has been detected at the GG sites separated from the nearest GG or AG sites by at least three base pairs. The AG sites separated from the nearest AG or GG sites by at least three base pairs do not denature the double helix locally when they are in the sequences puAG/pyTC. When they are in the pyAG/puTC sequences, the reactivity of osmium tetraoxide with the T residues complementary to the platinated A residues indicates either a distortion or an unpairing of the bases. The T residues within the sequences (CGT/GCA) react strongly with osmium tetraoxide. It is suggested that the distortion within these sequences is induced by adducts located further away along the DNA fragments, these sequences not being the major sites for the binding of *cis*-DDP.

Many studies suggest that both the cytotoxic and the antitumor activities of *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] are a consequence of its reaction with cellular DNA. Most of the adducts formed in the reaction of *cis*-DDP and DNA have been identified. Two major adducts arise from an intrastrand cross-link between two adjacent guanine residues and between the adjacent adenine and guanine residues. Minor adducts arise from intrastrand cross-links between two guanine residues separated by at least one nucleotide residue and from interstrand cross-links between two guanine residues (Roberts & Pera, 1983; Lippard, 1987; Reedijk, 1987; Eastman, 1987, and references cited therein).

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); dien-Pt, chlorodiethylenetriamineplatinum(II); DEPC, diethyl pyrocarbonate; CAA, chloroacetaldehyde; HA, hydroxylamine; I, d(CTCTCTTCTGTGTCTTCTCT); I*, the same oligonucleotide modified by *cis*-DDP at the GTG site; I-dienPt, the same oligonucleotide modified by dien-Pt at one G residue; II, d(AGAGAGAAGACACAGAAGAG); III, d(CTTCTCTTCTAGTCTTCTCT); III*, the same oligonucleotide modified by *cis*-DDP at the AG site; IV, d(GAGAGAAGACTAGAAGAGAA); V, d(CTCATCAGTCACTCT); V*, the same oligonucleotide modified *cis*-DDP at the AG site; VI, d(GAGAGTGACTGATGA); r_b, molar ratio of platinum residues per nucleotide; bp, base pair. Equimolar mixtures of the complementary oligonucleotides I and II, III and IV, and V and VI are respectively I + II, III + IV, and V + VI.

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