Inhibition of Electron Transport in Photosystem II by NH_2OH: Further Evidence for Two Binding Sites†

Mohanram Sivaraja and G. Charles Dismukes*
Department of Chemistry, Princeton University, Princeton, New Jersey 08544
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ABSTRACT: The reaction of hydroxylamine, a substrate analogue of the water-oxidizing complex (WOC), with spinach photosystem II (PSII) membranes has been further studied by using EPR spectroscopy to monitor the stepwise oxidation of donors and reduction of electron acceptors during successive low-temperature illuminations [Sivaraja, M., & Dismukes, G. C. (1988) *Biochemistry 27*, 3467–3475]. In addition to its well-known binding on the donor side of PSII, hydroxylamine binds in the dark with high affinity (K_D < 10 μM; <7 NH_2OH/PSII) to a site that structurally interacts with the primary electron acceptor FeQ_A^-. Binding in the dark to this acceptor site causes conversion of the normal g = 1.9 EPR signal for FeQ_A^- to g = 2.1 on the first turnover. This light-induced signal forms with or without exogenous electron acceptors and is maximized when Q_A is oxidized in the dark. The original g = 1.9 form recovers upon successive turnovers as the NH_2OH is consumed. The binding is blocked by addition of DCMU, which displaces the secondary electron acceptor Q_B, or by the presence of excess quinol and NH_2OH. These results indicate that the binding site for NH_2OH overlaps with or interacts with the binding site for Q_B. The EPR microwave power saturation of the g = 2.1 signal at 5.5 K is similar to that found for the endogenous ferrosemiquinone acceptors. These results indicate a structural change in the primary acceptor site upon binding NH_2OH, with no change in oxidation state of the iron or the semiquinone. In contrast, NH_2OH does not bind in the dark to PSII centers exhibiting the other major form of the primary acceptor, which exhibit the g = 1.82 EPR signal, since no change in the EPR signal is observed. We also find that the high-affinity binding of NH_2OH within the WOC produces no observable EPR-active products in the dark. Following illumination, this site is characterized by loss of formation of both of the EPR signals for photooxidized manganese in the S_2 state, without loss of photoreduction of the primary acceptor. We conclude that this binding site is closely associated with manganese, since there exists no blockage in the photooxidation of other donors like high-potential cytochrome b_{559} or signal II (^{160}Tyr·D_1 protein). A new EPR signal can be observed in both untreated and NH_2OH-treated PSII membranes extending over a 1000-G line width and centered at g = 2. It forms in the presence of an exogenous quinone acceptor upon multiple turnovers at 255 K in PSII membranes, or by 200 K illumination of NH_2OH-inhibited membranes. It is eliminated by DCMU and by removal of manganese upon treatment with excess NH_2OH. The possible identity of the species responsible for this new signal is discussed.

Analysis of the mechanism of photosynthetic water oxidation has been simplified by the use of substrate analogues such as NH_2OH, N_2H_4, and H_2O_2 which act as redox-active inhibitors of water oxidation (Bennoun & Joliot, 1969; Bouges, 1971; Cheniae & Martin, 1971; Velthuys & Kok, 1978). There is considerable circumstantial, but little direct, evidence suggesting that NH_2OH binds to manganese within the water-oxidizing complex (WOC) [reviewed by Radmer and Cheniae (1977), Dismukes (1986), and Babcock (1987)]. At low concentrations these inhibitors are now believed to function similarly by reversible two-electron reduction of the manganese complex. NH_2OH binds the strongest (K_D <10 μM; Sivaraja & Dismukes, 1988) compared to H_2O_2 (K_D ~1 mM; Mano et al., 1987; Frasch & Mei, 1987). Both produce a two-flash delay in O_2 production, third → fifth flash, associated with the $S_4 \rightarrow S_0$ transition (Bouges, 1971; Velthuys & Kok, 1978). In the case of NH_2OH it is also known that reduction of the WOC occurs at least as early as formation of the S_2 state on the first flash, and possibly even in the dark for the S_1 state. This is deduced from the two-flash retardation in the formation of the manganese multiline EPR signal for the S_2 state (Sivaraja & Dismukes, 1988). Evidence favoring S_2 as the target for the two-electron reduction instead of S_1 has come from Mn X-ray absorption edge experiments. These studies show that binding of NH_2OH causes no change in the edge energy when binding occurs in the dark S_1 state, while a decrease by 1.2–1.3 eV occurs only after illumination to form what would normally be the S_2 state (Guiles et al., 1986). By contrast, the S_2 state in untreated PSII samples exhibits an increase in edge energy by 1.1–1.2 eV. This decrease by 2.4 eV in the S_2 state edge energy in the presence of NH_2OH was attributed to the reduction of the manganese cluster by 2 equiv. This view is also supported by the considerably faster reactivity between NH_2OH and the S_2 state compared to the S_1 state (Andreasson et al., 1986; Sivaraja & Dismukes, 1988). Inhibition at this site is reversed by illumination, which consumes the NH_2OH. Radmer and Ollinger (1982) found that the two-electron reduction of the WOC by NH_2OH produces N_2 as the only gaseous product. The mechanism also produces two protons on the first flash which could come either from NH_2OH or from the WOC (Forster & Junge, 1986).

Binding to a lower affinity site occurs in the presence of an excess of NH_2OH, causing irreversible inhibition in the dark, due to the release of Mn (Cheniae & Martin, 1971; Yocum et al., 1981) and proteins from the WOC (Tamura & Cheniae,

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1985). There is evidence that this is a cooperative process in which 3 Mn/PSII are released per inactivating event, suggesting again the presence of a manganese cluster of 3 or 4 Mn/PSII (Sivaraja & Dismukes, 1988).

An analogous process occurs in the case of H_2O_2, where the two-flash delay in O_2 production observed under limiting H_2O_2 concentrations has been attributed to reduction of either S_2 to S_0 following the first flash or of S_1 to S_{-1} in the dark (Velthuys & Kok, 1978; Mano et al., 1987). Following a single turnover flash, a new reaction pathway becomes observable involving disproportionation of H_2O_2 to form O_2 and H_2O in the dark in a catalytic cycle between S_0 and S_2. Recent evidence has shown that this latter reaction can also be catalyzed by synthetic dimanganese complexes, which undergo two-electron oxidation by insertion of a μ-oxo bridge from H_2O_2, in contrast to mononuclear manganese complexes, which are unreactive (Mathur & Dismukes, 1987).

Thus the S_2 state of the WOC behaves as if it reduced reversibly to S_0 by both H_2O_2 and NH_2OH:

$$S_2 + H_2O_2 \rightarrow S_0 + 2H^+ + O_2$$

$$S_2 + 2NH_2OH \rightarrow S_0 + 2H^+ + N_2 + 2H_2O$$

Because H_2O_2 is thermodynamically unstable to disproportionation, it can reoxidize S_0 in a catalytic cycle, while NH_2OH can act only as a reductant and so is capable at best of further reduction of S_0:

$$S_0 + H_2O_2 + 2H^+ \rightarrow S_2 + 2H_2O$$

$$S_0 + xNH_2OH \rightarrow 3Mn^{2+}(\text{free}) + \text{Mn(bound)}$$

In addition to its reactions on the donor side of PSII, NH_2OH has recently been found to bind to a second high-affinity site (K_D <10 μM) that is distinguished by induction of a structural change in the "ferrosemiquinone" primary electron carrier, FeQ_A^-, as seen by a change in the g value for the EPR signal from 1.9 to 2.1 (Sivaraja & Dismukes, 1988). In this paper we try to resolve the various pathways for oxidation of PSII donors and reduction of acceptors in the presence of NH_2OH and further characterize the interaction it has with the electron acceptor site using EPR spectroscopy.

Materials and Methods

PSII membranes were prepared from spinach by the modified BBY procedure (Berthold et al., 1981; Ford & Evans, 1983) and stored in a 0.2 M sucrose buffer with 30% glycerol at −80 °C. Prior to use, these membranes were washed in the suspension buffer to remove the glycerol. The O_2 rate activities ranged from 300 to 350 μM O_2·(mg of Chl)$^{-1}$·h^{-1} at pH 6.5 with a Clarke type electrode at 20 °C (Hansatech Ltd). The electron acceptor used was 0.5 mM 2,5-dichloro-*p*-benzoquinone (DCBQ). This was added from a stock solution in dimethyl sulfoxide (DMSO); final DMSO concentration was 1–2%. All suspension buffers contained 0.2 M sucrose, 20 mM MES, and 15 mM NaCl. PSII membranes were initially washed with suspension buffer containing 2 mM ethylenediaminetetraacetic acid (EDTA). The number of chlorophyll molecules per PSI was taken as 225 Chl/PSII on the basis of determinations ranging between 200 Chl/PSII (Tamura & Cheniae, 1985) and 250 Chl/PSII (Ghanotakis et al., 1983; Cammarata et al., 1984).

Prior to treatment with NH_2OH the membranes were resuspended to a chlorophyll concentration of 4.0–4.5 mg/mL and dark-adapted for at least 30 min at 273 K in order to favor population of the S_1 state. NH_2OH was then added in the dark, and the suspension was gently stirred for 30 min. The exogenous acceptor was added next, and the membranes were transferred to EPR tubes and frozen in the dark.

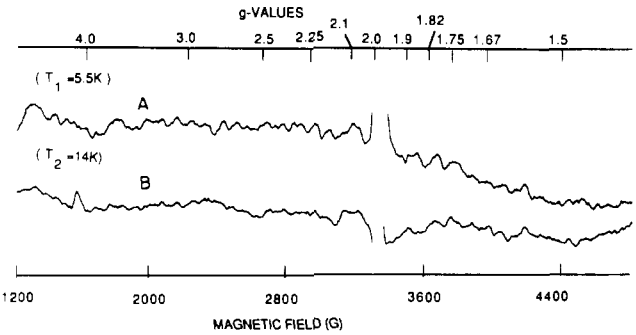


FIGURE 1: EPR difference spectra of NH_2OH-treated PSII membranes in the dark (7 NH_2OH/PSII) minus untreated PSII membranes in the dark. Sample temperature = 5.5 K (A) and 14 K (B). [Chl] = 4.0 mg/mL. Suspension buffer: 200 mM sucrose, 20 mM MES, and 15 mM NaCl at pH 6.5. Exogenous acceptor = 0.5 mM DCBQ. EPR conditions: microwave frequency, 9.22 GHz; microwave power, 40 mW; modulation amplitude, 32 G.

EPR spectra were obtained at 9.5 GHz on a Varian E-12 spectrometer operating with 10-kHz field modulation and fitted with an Oxford Instruments ESR-900 continuous-flow cryostat. The temperature at the sample was determined by using a gold–chromel thermocouple at the sample position. Two temperatures, 5.5 and 14 K, were chosen for EPR measurements to enable detection of paramagnetic centers having different temperature dependencies (Zimmerman & Rutherford, 1984; Rutherford & Zimmerman, 1984). These are appropriate for the g = 1.82 form of the ferrosemiquinone, and for oxidized cytochrome b_{559}, the manganese multiline and g = 4.1 signals and the g = 1.9 form of the ferrosemiquinone center, respectively. Difference spectra were obtained by using a Digital MINC-11 minicomputer and its 12-bit A/D module.

Quantitative estimates of the relative EPR signal yields following the indicated treatments are given in Table I. These are based on averages of the peak amplitudes for replicate samples.

Results

NH_2OH Binds but Does Not React with PSII Cofactors in the Dark S_1 State. Figure 1 shows the EPR difference spectra between PSII membranes incubated with 7 NH_2OH/PSII in the dark and untreated PSII membranes. Curve A was recorded at 5.5 K while curve B was recorded at 14 K. Two temperatures were used in order to optimize conditions for observing the various EPR signals, as described under Materials and Methods. There is no significant difference in the two samples at either temperature. The large difference signal at g = 2.0 arising from signal II and the small difference at g = 4.3 due to a rhombic ferric signal arise because of sample tube mismatch. At nonsaturating microwave powers (1 μW) the spectrum and amplitude for the free radical signal at g = 2 in both the control and the treated sample are indistinguishable. This lack of change means either that NH_2OH treatment in the dark does not change the population of EPR-active paramagnetic species or that the changes are undetectable under the conditions used.

Figure 1 also shows that the oxidized low-potential form of Cyt b_{559}, which is present in the dark in both the control and treated PSII membranes, is not reduced by addition of 7 NH_2OH/PSII, as evidenced by the lack of its characteristic rhombic signal at g_z = 2.95 and g_y = 2.26 (Bergstrom & Vanngard, 1982).

Table I: Percent Yield of Donor and Acceptor EPR Signals during Multiple Turnovers at Low Temperatures of Untreated and NH_2OH-Treated PSII Membranes

	donors					acceptors		
	S_2 multiline[a]	Cyt b (HP), $g = 3.0$[b]	$g = 2.0$, $\Delta H = 10$ G[c,f]	signal II[c]	$g = 2.0$, broad[d]	$g = 2.1$[e]	$g = 1.9$[e]	$g = 1.82$[e]
			(A) Control					
dark	0	0	0	75	0	0	0	0
200 K, illuminated	100	0	12	100	M[j]	0	100	100
warm to 255 K	100	0	0	100	M	0	0	0
200 K, illuminated	120	85	12	100	M	0	100	100
warm to 255 K	120	0	0	100	M	0	0	0
255 K, illuminated	20	0	0	100	100	0	80	85
warm to 255 K	20	0	0	100	0	0	70	80
			(B) 7 NH_2OH/PSII					
dark	0	0	0	75	0	0	0	0
200 K, illuminated	10	0	12	100	55[g]	100	0	100
warm to 255 K	10	0	0	100	0	0	0	0
200 K, illuminated	15	70	12	100	55	55	55	100
warm to 255 K	15	0	0	100	0	0	0	0
255 K, illuminated	20	0	0	100	75[h]	10	85	85
warm to 255 K	20	0	0	100	0	0	75	80
			(C) Mn Depleted[i]					
200 K, illuminated	0	100	10	0	0	0	100	100

[a] Signal to noise (S/N) for a single measurement = 10%; standard deviation (SD) based on three data sets = 3. [b] S/N = 15%; SD = 5. [c] S/N = 5%; SD = 0. [d] S/N = 20%; SD = 5.5. [e] S/N = 10%; SD = 10. [f] Given as percent area relative to signal II. [g] Variable from sample to sample; 20–75%. [h] Variable from sample to sample; 65–90%. [i] By treatment with 2 mM NH_2OH. [j] M indicates masked.

Initial Turnover in NH_2OH-Inhibited PSII at 200 K. Curves 1–3 of Figure 2 give the light minus dark difference EPR spectra for samples containing 7 NH_2OH/PSII and illuminated at 200 K. Each contains a different electron acceptor added 15 min after the NH_2OH was added in the dark: (curve 1) 2,5-dichlorobenzoquinone, (curve 2) ferricyanide, and (curve 3) no exogenous acceptor. These results show that the normal S_2 state EPR multiline signal that forms in the control (dotted curve) is absent in all three NH_2OH-treated samples, irrespective of the electron acceptor that is present. Second, one sees that the normal EPR signal at $g = 1.9$ for the primary electron acceptor is absent, and in its place a signal at $g = 2.1$ appears. The latter signal forms in all three samples, indicating that it arises from an endogenous electron carrier. The yield of this signal is maximized in curve 1, and weaker in curves 2 and 3, indicating that it increases in proportion of the effectiveness of the exogenous electron acceptor. Since the secondary acceptor site (Q_B) is depleted of plastoquinone (PQ) during the detergent extraction used to prepare the PSII membrane, this site is partially vacant (Figure 2, curves 2 and 3). As the extent of occupation of the Q_B site with an oxidized quinone is increased [Figure 2, curve 1 (PQ + DCBQ)], the yield of the $g = 2.1$ signal increases, indicating a positive correlation with the extent of oxidation of acceptors in the dark.

We examined the yields of the various donors and acceptors following successive low-temperature turnovers in order to establish what is undergoing oxidation and reduction in samples inhibited with a low concentration of NH_2OH. Initial turnover of the dark-adapted samples from Figure 1 was achieved by illumination at 200 K in the presence of the exogenous acceptor DCBQ. Figure 3 shows the light minus dark difference EPR spectra of untreated (A1 and B1) and NH_2OH-treated (A2 and B2) PSII membranes at 5.5 and 14 K (A and B, respectively). The results are similar to those in Figure 2 except that spectra shown in panel A were taken with conditions suitable for observing the $g = 1.82$ and $g = 1.9$ signals for the primary acceptor FeQ_A^-, while in panel B the conditions are better suited to observing the S_2 multiline EPR signal, the $g = 1.9$ FeQ_A^- signal, and oxidized Cyt b_{559}. The S_2 multiline EPR signal is formed in the untreated control

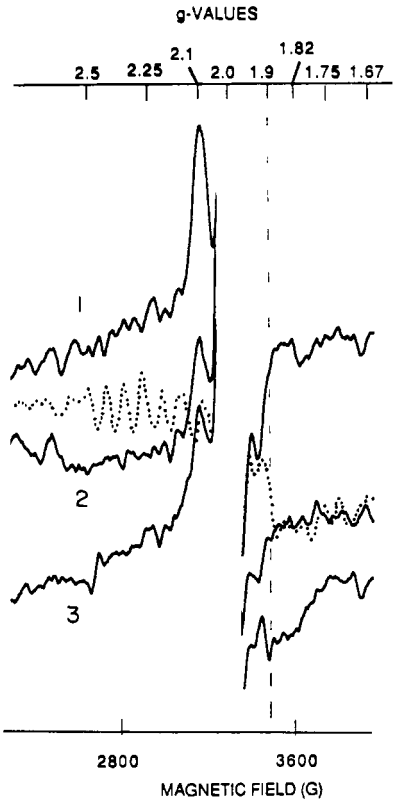


FIGURE 2: Light minus dark EPR difference spectra for NH_2OH-treated PSII membranes (7 NH_2OH/PSII) having different electron acceptors: DCBQ (curve 1), $K_3Fe(CN)_6$ (curve 2), and no exogenous acceptor (curve 3). For curve 3 the dark spectrum is the same as that used for curve 1. The dotted curve is the control for curve 2 showing the light minus dark trace for PSII membranes containing $K_3Fe(CN)_6$ as acceptor but with no NH_2OH. [Chl] = 5.5 mg/mL, [DCBQ] = 0.5 mM, and $[K_3Fe(CN)_6] = 1$ mM. Other conditions as in Figure 1.

sample (B1), but not in the NH_2OH-treated sample (B2), thus establishing saturation of binding to the high-affinity site in the WOC (Sivaraja & Dismukes, 1988). In the untreated membranes both the $g = 1.82$ (A1) and $g = 1.9$ (A1 and B1) signals for FeQ_A^- are formed by the illumination, while in the

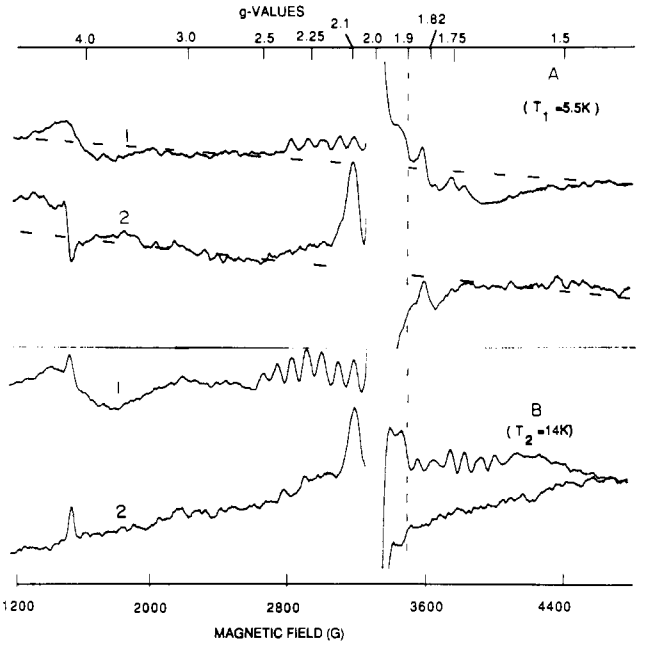


FIGURE 3: EPR difference spectra following initial turnover at 200 K in control (1) and NH_2OH-treated (2) membranes (7 nH_2OH/PSII). (A) Light minus dark EPR difference spectra of control (1) and NH_2OH-treated (2) PSII membranes after CW illumination at 200 K. The Chl concentration, suspension buffer, exogenous acceptor, and EPR conditions are the same as used in Figure 1. Sample temperature = 5.5 K. (B) Same as (A) except that the same temperature = 14 K.

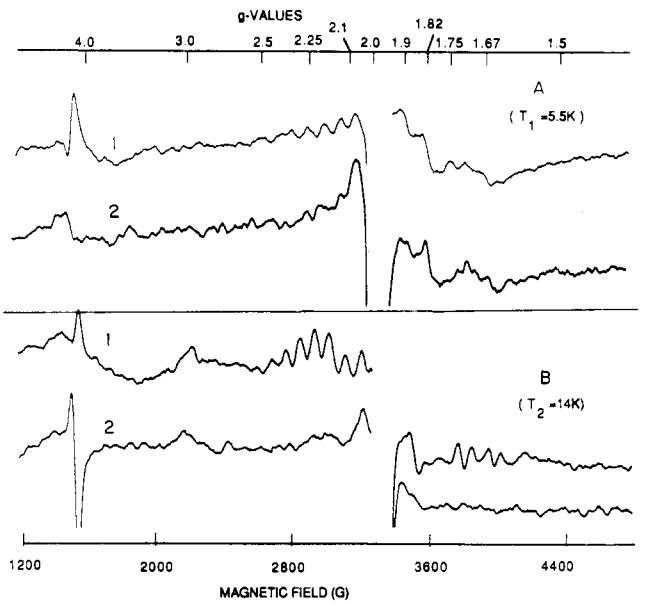


FIGURE 4: Effect of a second turnover by illumination at 200 K of control and NH_2OH-treated PSII membranes (7 NH_2OH/PSII). (A) Light minus dark EPR difference spectra of control (1) and NH_2OH-treated (2) PSII membranes after a second turnover at 200 K. Sample temperature = 5.5 K. (B) same as (A) except sample temperature = 14 K. The Chl concentration, suspension buffer, exogenous acceptor, and EPR conditions are the same as used in Figure 1.

NH_2OH-treated sample (A2 and B2) the $g = 1.9$ signal is not formed. As noted in Figure 2, the NH_2OH-treated sample exhibits the peak at $g = 2.1$ in place of the $g = 1.9$ signal. The line shape of this new signal requires that there must exist a feature of opposite polarity located at higher magnetic field strength. This feature appears to be located under the $g = 2$ region, where it is overlapped by the strong signal from signal II at $g = 2.00$.

Illumination at 200 K also forms a broad signal in the treated samples (A2 and B2) which cannot be seen in the untreated sample due to the large multiline signal in this region that forms in the S_2 state. It exhibits a weak featureless spectrum beginning at 2600 G and is centered at around $g = 2$. The yield of this broad signal varied a lot from sample to sample on the first turnover.

High-potential Cyt b_{559} is not photooxidized by the first illumination at 200 K in both untreated (B1) and NH_2OH-treated PSII membranes (B2), since we see no light minus dark difference signal at $g = 3.05$ in panel B (Bergstrom & Vanngard, 1982; Crowder et al., 1982). This same result has been observed for untreated PSII membranes by de Paula et al. (1985).

In these dark-adapted samples illumination at 200 K increases the yield of signal II_{slow} by an additional 25% of the maximum signal in both control and NH_2OH-treated samples. Illumination also induces formation of a minor free radical signal at $g = 2.00$ in both samples (10-G spectral width and about 0.1 spin relative to the maximal yield of signal II_s) (data not shown). These changes have been seen previously by several groups and reflect, respectively, the fraction of centers in the dark S_1 state having reduced signal II_s centers and disconnected PSII centers having no other available donors.

In agreement with earlier work on untreated PSII membranes (de Paula et al., 1985), the major EPR-detectable photooxidized donor seen on the first turnover is the manganese center, with the electron acceptor being FeQ_A. Our results on the quantitative yields of turnover are summarized in Table I and represent the average of three or more data sets. For NH_2OH-treated samples photoreduction of the acceptor shows up as both the normal yield of the $g = 1.82$ form of FeQ_A^- and the new signal at $g = 2.1$ (Table I). However, the identity of the photooxidized donor is not clear. The only observable signal that could be a candidate for the electron donor is the broad signal at $g = 2$. However, this forms in variable yield and so cannot be the principle electron donor, if due to a donor species at all.

Second Turnover of NH_2OH-Inhibited PSII at 200 K. A second turnover at 200 K was performed to test for reversal of inhibition. This was achieved by warming the previously illuminated samples to 255 K for 1.5 min in the dark, to allow reoxidation of FeQ_A^-, followed by refreezing and illumination again at 200 K. Confirmation that warming to 255 K in the dark after the initial 200 K illumination reoxidized FeQ_A^- was taken from the disappearance of the ferrosemiquinone signals from both the control sample ($g = 1.82$ and $g = 1.9$) and the NH_2OH-treated samples ($g = 1.82$) (data not shown). It was also observed that both the $g = 2.1$ and the broad $g = 2$ EPR signals seen in NH_2OH-treated samples disappeared upon warming to 255 K in the dark (data not shown).

Figure 4 shows representative light minus dark EPR difference spectra for the second turnover of untreated (A1 and B1) and NH_2OH-treated samples (A2 and B2) at 5.5 and 14 K (A and B, respectively). The average change in yield of the signals for three or more data sets is given in Table I. In the control sample the multiline signal is present with about 20% greater yield than found for the first turnover (B1). The $g = 1.82$ and $g = 1.9$ signals are again formed in the control sample with the same yield (A1). In the NH_2OH-treated sample we again see no formation of the multiline signal (B2), a small decrease in yield of the $g = 1.82$ signal (A2), appearance of a small $g = 1.9$ signal (B2), a decrease in the yield of the $g = 2.1$ signal, and reappearance of the weak broad signal (A2). The second turnover now gives a signal at $g = 3.0$ due to oxidized high-potential Cyt b_{559} in both control (B1)

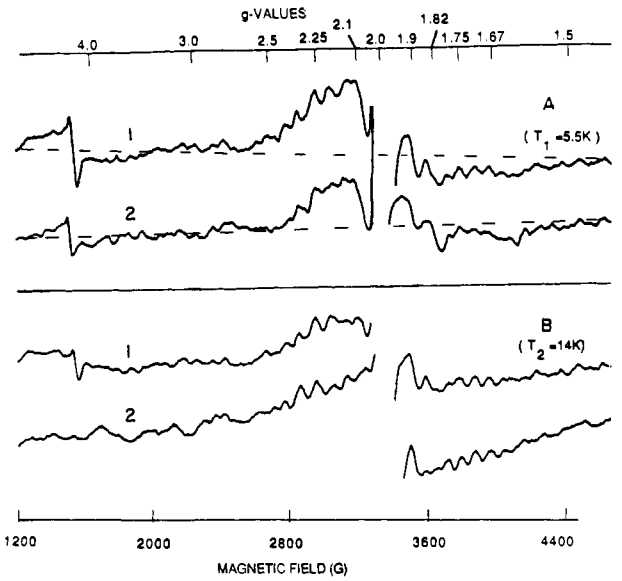


FIGURE 5: Effect of illumination at 255 K on control and NH_2OH-treated PSII membranes (7 NH_2OH/PSII). (A) Light minus dark EPR difference spectra for the control (1) and NH_2OH-treated (2) PSII membranes from Figure 3 after dark readaptation and CW illumination at 255 K. Sample temperature = 5.5 K. (B) Sample temperature = 14 K. The Chl concentration, suspension buffer, exogenous acceptor, and EPR conditions are the same as for Figure 1.

and NH_2OH-treated (B2) samples.

These results indicate that the major observable donor for the second turnover is the high-potential Cyt b_{559} in both the control and the NH_2OH-reduced sample (Table I). The acceptors in this case are distributed between the $g = 1.82$ and $g = 1.9$ forms of FeQ_A^- and the $g = 2.1$ species. The loss in yield of the $g = 2.1$ signal on the second turnover is accompanied by an increase in the yield of the $g = 1.9$ signal, such that the sum of the relative amplitudes is undiminished (Table I).

Multiple Turnovers in PSII by Illumination at 255 K. A third turnover at 200 K did not bring back the S_2 multiline signal in the NH_2OH-treated sample, owing to the presence of a thermal block that can be overcome by flash illumination at 277 K (Sivaraja & Dismukes, 1988). Therefore, illumination at a higher temperature, 255 K, was carried out to see if this could be overcome. Figure 5 shows the light minus dark difference EPR spectra after illumination at 255 K of untreated (A1 and B1) and NH_2OH-treated (A2 and B2) PSII membranes. Both dark-adapted samples and the preilluminated samples from Figure 3 that were dark-adapted gave similar results with illumination at 255 K. With the higher temperature illumination both the NH_2OH-treated and the control samples exhibit comparable yields of the FeQ_A^- signal at $g = 1.9$ and at $g = 1.82$, which is about 85% of the amplitude from untreated PSII membranes illuminated at 200 K (Table I). Accompanying this reappearance of the $g = 1.9$ acceptor signal in NH_2OH-treated samples, there was a parallel reduction of the $g = 2.1$ signal. Illumination at this higher temperature also appreciably increased the amplitude of the $g = 2$ broad signal in both samples, with the untreated sample exhibiting a 25% greater amplitude than the NH_2OH-treated sample. This greater amplitude allows a clearer view of the broad spectral shape of this signal. On the low-field side the absorption begins at about 2000 G and rises to the peak at 3000 G. The amplitude of the multiline signal has decreased to 20% of the first turnover amplitude in the control sample, while it has increased to this same level in the

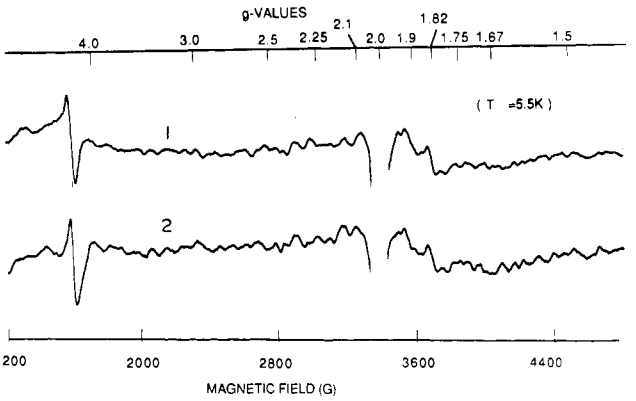


FIGURE 6: Effect of incubation in the dark for 1.5 min at 255 K after CW illumination at 255 K. The illuminated samples were treated the same as those used in Figure 5. The spectra are the difference between samples treated in this way and previously unilluminated samples. (1) Control PSII membranes; (2) 7 NH_2OH/PSII membranes. Chl concentration = 4.5 mg/mL; other conditions are as in Figure 1. The temperature is 5.5 K.

NH_2OH-treated sample. Neither photooxidized high-potential Cyt b_{559} nor the 10-G-wide free radical signal are formed. Signal II_s is also not formed beyond the initial 25% seen with 200 K illumination. These results are summarized in Table I.

The principal result given in Figure 5 is that when multiple turnovers are allowed by illumination at 255 K a new steady state is reached. This causes the formation of both the $g = 1.9$ and $g = 1.82$ FeQ_A^- acceptors as the major pathway for reduction, along with formation of the broad $g = 2$ signal in both samples. Since these are the only stably induced EPR signals, the broad $g = 2$ signal is a leading candidate for a photooxidized donor responsible for reduction of the acceptors. Since multiple turnovers are allowed at this temperature of illumination, it is also possible that EPR-silent donors are the source of the electrons.

Recombination Pathways in the Dark following Multiple Turnovers in PSII. Figure 6 shows the EPR difference spectra recorded at 5.5 K for samples warmed in the dark to 255 K for 1.5 min after illumination at 255 K in both untreated (curve 1) and NH_2OH-treated PSII membranes (curve 2). This dark incubation causes the broad $g = 2$ signal to disappear, while the acceptor signals at $g = 1.9$ and 1.82 do not decrease in either sample. The stability of the acceptor signals is in contrast to their behavior in samples warmed after 200 K illumination (single turnover conditions).

Effect of DCMU on Multiple Turnovers. Experiments were performed by using the electron transport inhibitor DCMU to restrict electron turnover. Since DCMU inhibits by displacement of Q_B, it restricts charge separation to a single electron turnover under normal circumstances (Velthuys & Amesz, 1974; Renger, 1976). Earlier we showed that DCMU addition prior to NH_2OH addition blocked formation of the $g = 2.1$ and loss of the $g = 1.9$ signals that occur in the absence of DCMU (Sivaraja & Dismukes, 1988). We have since examined the reversal of these additions and find the same result (data not shown). This indicates that in the dark S_1 state binding of NH_2OH to the acceptor site is in competitive equilibrium with the binding of DCMU. Again we see that the NH_2OH acceptor site overlaps or interacts with the DCMU site which is located in the Q_B pocket.

Figure 7 shows an extension of this type of experiment except with illumination at 255 K where multiple turnovers are not thermally inhibited. The PSII samples were pretreated with 150 μM DCMU in the dark. The spectra shown are

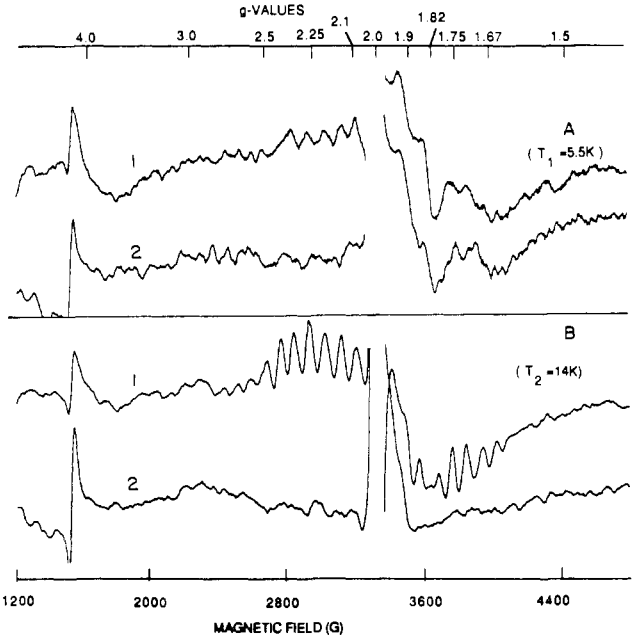


FIGURE 7: Effect of DCMU on multiple turnover at 255 K in control and NH_2OH-treated (7 NH_2OH/PSII) PSII membranes. (A) Light minus dark difference EPR spectra for control (1) and NH_2OH-treated (2) PSII membranes pretreated with 150 μM DCMU in the dark and illuminated at 255 K. Sample temperature = 5.5 K. (B) Same as (A) except sample temperature = 14 K. Chl concentration = 4.5 mg/mL. All other conditions were as in Figure 1.

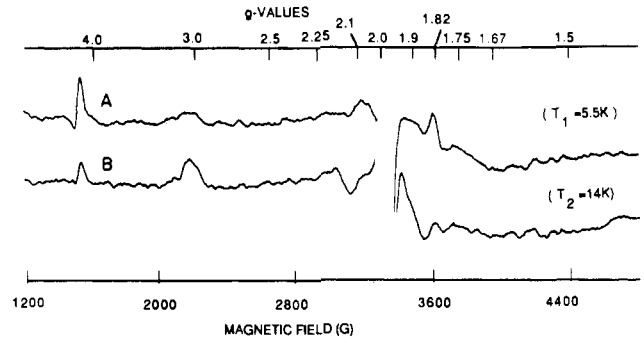


FIGURE 8: Effect of 200 K illumination of PSII membranes pretreated with 2 mM NH_2OH in the dark and then washed with 2 mM EDTA. Light minus dark difference EPR spectra are shown at sample temperature = 5.5 K (A) and 14 K (B). [Chl] = 4.0 mg/mL. All other conditions were as in Figure 1.

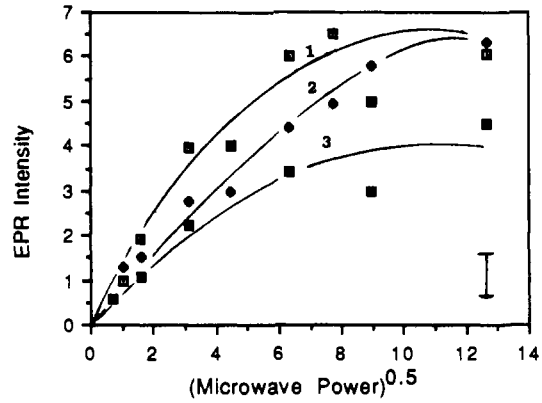


FIGURE 9: EPR microwave power dependence of the intensity of the g = 1.82 (curve 1), g = 1.9 (curve 2), and g = 2.1 (curve 3) signals. The g = 1.82 and g = 1.9 signals were examined in untreated PSII membranes illuminated at 200 K, and the g = 2.1 signal was monitored in PSII membranes pretreated with 7 NH_2OH/PSII in the dark and illuminated at 200 K. EPR conditions are the same as in Figure 1. Sample temperature = 5.5 K. The data were arbitrarily scaled to different relative amplitudes to facilitate viewing.

representative light minus dark differences. The average yields of the light-induced signals are given in Table I.

The S_2 multiline signal is formed in the control membranes (B1) at a yield that is comparable to but usually slightly less than that for illumination at 200 K without DCMU, once the data are normalized. No multiline signal forms in the NH_2OH-treated membranes (B2). The ferrosemiquinone signals at g = 1.82 and g = 1.9 are formed in both samples essentially to the sample extent (A1 and A2). The broad g = 2 signal, which was previously shown to form upon ilumination at 255 K in both the control and the NH_2OH-treated samples (Figure 5), is not formed in samples preincubated with 150 μM DCMU. This indicates either that multiple turnovers are required for its formation or that DCMU prevents the binding of exogenous quinone needed for formation of this signal. As we previously reported for 200 K illuminations, the g = 2.1 signal is also absent in the NH_2OH-treated sample that is preincubated with DCMU and illuminated at 255 K (A2). In its place the g = 1.9 form of FeQ_A^- appears. Cyt b_{559} is not photooxidized in either sample (B1 and B2), as expected if the number of turnovers is limited to one.

Single Turnover of Manganese-Depleted PSII Membranes. PSII membranes were pretreated with a large excess of NH_2OH (2 mM) in the dark, which releases manganese from the WOC and reduces signal II. These samples were frozen in the dark and illuminated at 200 K to see if the species producing the acceptor signal at g = 2.1 and the broad signal at g = 2 would still form. EDTA (2 mM) was also present in these samples in order to chelate Mn^{2+} released by the NH_2OH treatment, which otherwise gives a large six-line EPR spectrum at g = 2. Figure 8 shows the light minus dark difference spectra recorded at 5.5 K (A) and 14 K (B). The usual g = 1.82 (A) and g = 1.9 (B) acceptor signals are formed along with the photooxidized donor high-potential Cyt b_{559} seen at g_z = 3.0 and g_y = 2.2 (B). Little or no g = 2.1 signal forms upon illumination under these conditions. Furthermore, when these samples are examined at low microwave power, there is no evidence that signal II_s is reoxidized or that the free radical signal at g = 2.00 forms to any greater extent than for untreated samples (Table I).

Ghanotakis and Babcock (1983) have shown that NH_2OH at 1–2 mM concentration reversibly blocks electron transport at 277 K between Z and P_{680}^+. They observed transient formation of a P_{680}^+ radical which recombined with Q_A^- under multiple-flash conditions where oxidation of high-potential Cyt b_{559} should have occurred if it does not require Z^+. Our data show that a stable P_{680}^+ radical is not formed after 200 K illumination of PSII membranes treated with excess NH_2OH, as is expected at this temperature. In the absence of the endogenous manganese center, high-potential Cyt b_{559} becomes the terminal donor responsible for Q_A reduction at 200 K. Thus the block that occurs at 277 K between Z and P_{680}^+ is not effective in abolishing photooxidation of Cyt b_{559}, which must, therefore, be coupled directly to P_{680}.

Microwave Saturation of the g = 2.1 Signal. Figure 9 shows the microwave power dependence of the g = 1.82 (curve 1), g = 1.9 (curve 2), and g = 2.1 (curve 3) EPR signals at 5.5 K. The amplitudes have been normalized to different relative amplitudes to facilitate viewing of the data. The three signals have similar power saturation behavior, which differs greatly from that for typical free radicals like signal II. They can be saturated only at very low temperatures. Accordingly, the species giving rise to the g = 2.1 signal must be associated with a rapidly relaxing paramagnetic center, analogous to the

ferrosemiquinone FeQ_A^-. Because of this the EPR intensity of the $g = 2.1$ signal would not be a suitable indicator of spin concentration when compared to that of simple free radicals.

Influence of NH_2OH on Electron-Transfer Pathways for Oxidation and Reduction in PSII. Table I summarizes the yields of the various EPR signals following the illumination and dark warming cycles described in the previous figures. They are grouped as donor or acceptor signals according to earlier published work. The new signal at $g = 2.1$ is placed in the acceptor category according to the evidence given here. We are uncertain of the origin of the broad $g = 2.0$ signal; hence it is placed in the table without a heading. These data are the means of replicate experiments as indicated in the table footnotes and so do not refer exclusively to the results given in the figures. They represent relative amplitudes of peak heights and do not take into account line-shape changes that may occur at the different temperatures. The maximum observed yield of each signal is set at 100%.

The NH_2OH-treated samples contain 7 NH_2OH/PSII, since this was just sufficient to inhibit formation of the S_2 multiline EPR signal without appreciable release of Mn from the WOC. This ensures saturation of the high-affinity binding sites within the WOC and the ferroquinone complex, but still retains functional manganese centers that can be reactivated by illumination at room temperature (Sivaraja & Dismukes, 1988).

The behavior of the electron acceptors is the simplest to follow. Comparing Table IA for untreated samples to Table IB for NH_2OH-treated samples, we see that NH_2OH induces a complete loss of the $g = 1.9$ signal for FeQ_A^- on the first turnover, and this is accompanied by the formation of the $g = 2.1$ signal. On the second turnover about 50% of the $g = 2.1$ signal is lost, but this is replaced by an equal relative yield of the $g = 1.9$ signal. Illumination at 255 K, which allows multiple turnovers, causes almost complete loss of the $g = 2.1$ signal, which is compensated by a parallel increase in the $g = 1.9$ signal. These results can be understood if NH_2OH binds with high affinity near the acceptor site in PSII membranes which exhibit the $g = 1.9$ form for FeQ_A^- and convert this to a new form absorbing at $g = 2.1$. Following one, two, and multiple turnovers, at temperatures where diffusion is suppressed, there is progressive conversion to the original $g = 1.9$ form, suggesting that displacement or consumption of NH_2OH occurs upon turnover.

In contrast to this behavior, the $g = 1.82$ form of FeQ_A^- is unaffected in both treated and untreated samples for all conditions studied here. Evidently this spectral form arises from a site which is resistant to binding by NH_2OH. Furthermore, incubation with the herbicide DCMU increased the yield of the $g = 1.82$ signal in both treated and control samples, further indicating that PSII membranes with this type of acceptor do not bind NH_2OH at the acceptor site.

At 255 K, where multiple turnovers are allowed, illumination of both treated and untreated samples produces about the same yield of the $g = 1.9$ and 1.82 signals. However, unlike the single- and double-turnover experiments at 200 K, subsequent incubation in the dark at 255 K for both samples does not cause any decay of these signals. This indicates that both reoxidation by Q_B and recombination with a donor are blocked in the dark at 255 K.

On the donor side NH_2OH blocks the low-temperature formation of the normal S_2 state as seen by the loss of the multiline signal on both the first and second turnovers. Earlier we reported an experiment designed to produce a triple turnover at 200 K which failed to see any recovery of this signal or the $g = 4.1$ signal (Sivaraja & Dismukes, 1988). Table I shows that under multiple-turnover conditions at 255 K about 20% recovery of the maximum yield of the multiline signal is observed in both NH_2OH-treated and control membranes. Thus the blockage that exists at 200 K is not evident once illumination at 255 K is performed.

Parallel to this, the broad $g = 2$ signal appears in 55% yield on both the first and second turnovers in the NH_2OH-treated samples. It is not possible to see if this signal is present in the untreated membranes due to overlap from the strong multiline signal (M indicates masked in Table I). In the NH_2OH-treated samples this signal amplitude increases to the maximum value of 75%. The maximum amplitude in untreated membranes (100%) is also produced by illumination at 255 K.

Table I shows that the yields for photooxidation of high-potential Cyt b_{559}, signal II_s, and the $g = 2.00$ free radical are unaltered by the addition of 7 NH_2OH/PSII. High-potential Cyt b_{559} becomes an effective donor only on the second turnover at 200 K, and this pathway is not altered in the NH_2OH-treated samples. Thus these donors behave as though they are photooxidized by the reaction center at sites that are unaffected by the reversible reduction of manganese in the WOC by NH_2OH. Once the manganese is removed by treatment with 2 mM NH_2OH, high-potential Cyt b_{559} becomes the only donor observable at 200 K, apart from the usual minor free radical signal. These results are not unexpected on the basis of previous studies which place these donors on side paths not directly involved in electron transfer between the reaction center and the WOC.

Discussion

Origin of the $g = 2.1$ EPR Signal—NH_2OH Reactions with the Donor Side of PSII. The data in Figure 1 show that at concentrations where the high-affinity binding sites are occupied by NH_2OH (7 NH_2OH/PSII), there are no EPR-observable changes in any of the dark signals nor formation of new signals. This suggests either that there is no reaction in the dark or that the reaction products are not EPR detectable. Only following illumination are changes revealed between the untreated and NH_2OH-treated samples.

The data in Table I reveal a reciprocal correlation in the appearance of the $g = 1.9$ and 2.1 signals. We recently reported another test of this correlation in which the inhibitor DCMU was added to block access to the Q_B site (Sivaraja & Dismukes, 1988). The results demonstrated that if DCMU is added before addition of NH_2OH, it blocks the appearance of the $g = 2.1$ signal and retains the $g = 1.9$ signal. As reported here, we also see that the reverse addition of NH_2OH followed by DCMU in the dark produces the same result with regard to the acceptor site for NH_2OH binding. Thus the cumulative evidence supports the view that the $g = 2.1$ signal represents a new spectral form of the FeQ_A^- species that is created by the reversible binding of NH_2OH to a site (designated the A site, for acceptor side) that is accessible only through the Q_B binding site in PSII membranes exhibiting the $g = 1.9$ signal. In contrast, the $g = 1.82$ signal remains unaltered throughout this conversion and so behaves as though it does not bind NH_2OH or is unreactive to NH_2OH. This blockage of the Q_B site by DCMU does not reverse the loss in the multiline signal that NH_2OH causes, indicating that there is a second high-affinity site for NH_2OH binding (designated the O site, for oxidizing side) which is more closely associated with manganese in the WOC.

From its weak microwave power saturation seen at 5.5 K it is clear that the $g = 2.1$ signal is not due to a simple free

radical spin, which would be strongly saturated. The evidence indicates that this signal is due to a structurally altered FeQ_A^- acceptor species. Since the line shape of the $g = 2.1$ signal is overlapped by the $g = 2$ signal, it is not possible to integrate the signal. Nor is it apparent that this would provide an estimate of spin concentration since, like the $g = 1.9$ and 1.82 acceptor signals, this signal probably represents population of one sublevel of a multisublevel spin system (Dismukes et al., 1984; Butler et al., 1984).

The existence of two spectral forms for the primary ferrosemiquinone acceptor at $g = 1.82/1.65$ and $1.9/1.64$ has been known for several years both in bacteria (McElroy et al., 1970; Leigh & Dutton, 1972) and in algae and plants (Nugent et al., 1981; Rutherford & Zimmerman, 1984). In the case of spinach, these forms are interconverted reversibly by pH changes, with the $g = 1.9$ form being expressed above pH 6 and in intact chloroplasts (Rutherford & Zimmerman, 1984).

In a study of the line shape of the ferrosemiquinone EPR signals in the bacterium *Rhodobacter viridis* a spectrum with rhombic symmetry exhibiting three apparent g values of 2.11, 1.84, and 1.65 has been observed in whole cells but is greatly altered in chromatophores (Dismukes et al., 1984). The signal appears under reduction with dithionite and so corresponds to the oxidation state $Q_A^-FeQ_B^{2-}$. The $g = 2.11$ component of this signal was not present in chromatophore membranes and so is sensitive to membrane disruption. A different line shape is obtained for the state $Q_AFeQ_B^-$, where there is little or no $g = 2.11$ feature present. In no case has a resonance with $g > 2.0$ been observed previously independent of the other resonances. A theoretical description of these resonances has been given previously as due to the electron spin exchange interaction between a semiquinone (spin $S = {}^1/_2$) and a rhombic symmetry Fe^{2+} (high spin, $S = 2$) (Butler et al., 1984; Dismukes et al., 1984).

If the action of low concentrations of NH_2OH in PSII were to reduce Q_B to $(QH_2)_B$, then the shift of the $g = 1.9$ signal to 2.1 might represent the counterpart of the line-shape change observed in bacteria for the $g = 1.84$ signal upon reduction of $Q_AFeQ_B^-$ to $Q_A^-FeQ_B^{2-}$. There is, however, no evidence to support an identification of the $g = 1.9$ signal in PSII with the $Q_AFeQ_B^-$ state, which is not expected to form upon illumination at 200 K since $Q_A^- \rightarrow Q_B$ electron transfer is thermally activated (Wraight et al., 1986). The simpler alternative of a structural change of the environment around FeQ_A^- is therefore the only explanation that appears to fit all of the observations. This alteration may be induced by changes brought about by NH_2OH binding directly within the Q_A site, within the iron site, or within the Q_B site, which interacts with the Q_A site via the histidine–Fe–histidine bridge anticipated from the analogy to the bacterial reaction center structure (Deisenhofer et al., 1985).

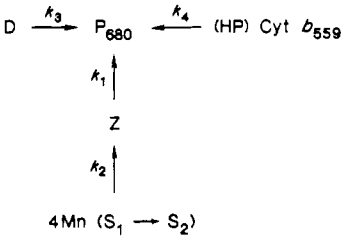
The possibility that the $g = 2.1$ signal arises from the split doublet signal for the state, $Ph^-Q_A^-FeQ_B^{2-}$, in which pheophytin (Ph) is reduced to the anion radical, will be considered next. This doublet signal arises from the magnetic coupling between Ph^- and Q_A^- (Klimov et al., 1980) and is formed under strong reducing conditions during illumination. This has a splitting of 45–60 G centered about $g = 2.00$ and so has no peak extending into the $g = 2.1$ region. By contrast, the $g = 2.1$ signal appears about 140 G below the region where the doublet would be centered. Also, the $g = 2.1$ signal disappears under stronger reducing conditions at high concentrations of NH_2OH, conditions that should be more favorable for reducing pheophytin. Thus both the spectral width and the conditions for formation of the $g = 2.1$ signal are inconsistent with it being due to pheophytin reduction in the state $Ph^-Q_A^-FeQ_B^{2-}$.

NH_2OH binding to the A site at low concentrations enables formation of the $g = 2.1$ form of FeQ_A^-, whereas at high concentrations (2 mM) the normal $g = 1.9$ signal forms (Figure 8). The reason for this reversion to the normal signal is not clear. One possible idea that fits in with the DCMU blockage experiment is that in the presence of a high concentration of the quinol form of the acceptor (DCBQ + NH_2OH) the Q_B site may be occupied with the quinol $(QH_2)_B$, thus blocking the NH_2OH influence on the $g = 1.9$ signal of the primary acceptor. By contrast, when the Q_B site is occupied by an oxidized quinone, we see maximum conversion of the $g = 1.9$ to $g = 2.1$ signals (Figure 2).

Single-Electron Turnovers in Untreated PSII Membranes. Brudvig et al. (1983) demonstrated that the one-electron oxidation of the S_1 state to the S_2 state and reduction of FeQ_A constitute the major light reaction in PSII membranes at 200 K. Our results agree with theirs. In addition, we find that signal II_s acts as a minority donor in 25% of centers. This appears to be in competition with the oxidation of S_1 to S_2, since the second turnover results in an additional 20–25% increase in the S_2 multiline signal as previously noted (Damoder et al., 1986). Thus, after the first turnover the 25% centers containing the newly photooxidized signal II_s donor also contain unoxidized Mn clusters (S_1). These are oxidized only on the second turnover (Table I). A slow oxidation of reduced signal II_s by the photooxidized Mn cluster in the S_2 state at 210 K has been previously observed (Kawamori et al., 1987). Styring and Rutherford (1987) have recently shown that the manganese complex is oxidized by the species yielding signal II_s, attributed to a tyrosine radical D^+ (^{160}Tyr of D_1 protein; Debus et al., 1988), when the former is placed in the S_0 state by three flashes at 277 K. Thus D and the Mn cluster are in redox equilibrium. The picture that emerges from this behavior is consistent with a physiological role for D^+ as a relatively long-lived storage site for 1 oxidizing equivalent in a sequestered (unreactive) environment. This could be capable of oxidizing the WOC under conditions of low light flux where oxidizing equivalents are unavailable and reduction of the S states could lead to inactivation. Indeed, it has been shown that D^+ functions to oxidize the reduced quinol acceptors which, being diffusable in the membrane, are capable of short-circuting water oxidation by reduction of manganese (Damoder et al., 1986). Rutherford and Styring (1987) have extended this view to include a possible role for D^+ in the so-called photoactivation process in which free Mn^{2+} religates to the dissociated WOC.

High-potential Cyt b_{559} becomes an available donor at 200 K only on the second turnover in 80% of centers. No limitation on the acceptor side is seen. It thus appears as though high-potential Cyt b_{559} functions as a low-temperature donor only after oxidation of S_1 and signal II_s. This extends the earlier results by Knaff and Arnon (1969) demonstrating oxidation of Cyt b_{559} below 150 K in untreated membranes. We have normalized to 100% the yield of high-potential Cyt b_{559}^+ formed by illumination of PSII membranes at 200 K that were treated with 2 mM NH_2OH in the dark. We found the intensity of the light-induced $g_z = 3.0$ peak (Crowder et al., 1982) to be identical with the intensity of the $g_z = 2.95$ peak for the low-potential form of Cyt b_{559} present in dark-adapted, untreated PSII membranes (Bergstrom & Vanngard, 1982). Thus the relative yields of the high-potential and low-potential forms are the same in untreated membranes, consistent with absolute yield measurements showing 2 Cyt b_{559}/PSII (de

Scheme I



Paula et al., 1985). The picture that emerges for two consecutive one-electron turnovers at 200 K of untreated PSII membranes appears fairly straightforward and can be represented by Scheme I, with rate constants $k_1 > k_2 > k_3 > k_4$ at 200 K.

Origin of the Broad g = 2 EPR Signal in PSII. The maximum yield of the broad $g = 2$ signal seen with illumination at 255 K in untreated membranes appears to involve three turnovers, as judged from the yield and stability of the FeQ_A^- EPR signals. Both the $g = 1.82$ and the $g = 1.9$ signals are formed in 80% yield by illumination at 255 K and are stable against decay in the dark at 255 K for 2 min, unlike their disappearance in samples warmed following single-turnover illuminations at 200 K. This can be understood if three electrons are transferred to form $Q_A^-FeQ_B^{2-}$, a state that should be stable at 255 K due to the slow reoxidation of Q_B^{2-} at this temperature. This view of the electron transfer supports an assignment of the broad $g = 2$ signal with a species in the S_0 state, assuming that all centers have advanced to the same S state. This latter result seems to be largely true as seen by the nearly unaltered yield of the reduced acceptors (80–85%, Table I).

The broad signal was also observed in a significant fraction of centers following a single-turnover illumination at 200 K in NH_2OH-treated samples, although the amplitude was highly variable (20–70%). The average amplitude on the second 200 K illumination was the same as on the first turnover, but more reproducible. The yield of the broad signal did not increase with the age or the number of freeze/thaw cycles of the PSII membranes. The signal was strongest in preparations that exhibited the highest O_2 evolution activity. Hence, it behaves as though it is an important component in active samples. The number of spins represented by the broad $g = 2$ signal could not be determined because it is overlapped by several other signals below $g = 2$, which makes integration unfeasible.

The appearance of the broad signal following 200 K illumination in NH_2OH-treated samples also supports an association with the S_0 state. Since NH_2OH reduces the WOC by two electrons, an S_0 type state (S_0^*) should result from a single-turnover illumination at 200 K. This should differ from the normal S_0 state in that O_2 is not a product and the reaction products of NH_2OH oxidation may also influence the EPR properties. This apparent association with the S_0 state does not help to distinguish the signal as due to a donor or an acceptor of electrons.

There is a major discrepancy in assigning the signal to a major donor in the S_0 state. If the NH_2OH-treated samples are first incubated with DCMU, then the broad signal does not form upon illumination, neither at 200 K nor at 255 K even though charge separation to form the S_0^* state occurs. The blockage of the broad $g = 2$ signal by DCMU could have two explanations—either its formation requires access through a vacant Q_B site or multiple turnovers are needed to produce it. If the former is the case, then the signal might arise in some centers from the binding of an extra exogenous acceptor molecule via the Q_B pocket at a site close to Q_A, where it could undergo photoreduction even at low temperature.

An assignment of the broad $g = 2$ signal to an endogenous reduced acceptor species in the S_0 state in the majority of centers is not supported by the yields for the normal FeQ_A^- signals at $g = 1.9$ and 1.82. These are formed in 80 and 85% yields, respectively, in untreated samples illuminated at 255 K, and so appear not to be substantially replaced by the broad signal which forms in maximal yield under these conditions. Another difference is that the broad signal decays in the dark at 255 K after illumination at 255 K, unlike the acceptor signals. This suggest that the broad signal may arise in centers that do not give rise to the normal ferrosemiquinone EPR signals. Thus we propose that the broad signal could be reconcilled with the presence of a new electron acceptor in *some* reaction centers that can undergo photoreduction at 200 K. This acceptor would have to bind to a site whose reduction or binding is blocked by the binding of DCMU to the Q_B site. The identity of this acceptor is unknown, but we suggest that it could be due to exogenous DCBQ bound within the reaction center. Indeed, X-ray crystallographic studies of *R. viridis* reaction centers crystallized in the presence of excess ubiquinone have found as many as six possible binding sites (H. Michel, private communication).

Since charge separation requires both an electron donor and an acceptor, it could also be argued that the broad $g = 2$ signal may be due to photooxidation of an electron donor in some centers. If we consider now the possibility that in some centers (15–20%) multiple turnovers could occur at 200 K in the presence of the exogenous acceptor DCBQ, we could attribute the broad $g = 2$ signal to photooxidation of the extra donor, other than S_1, that gets oxidized in this minority of centers. We are unable to distinguish between these alternative sites for the broad $g = 2$ signal at present.

Added in Proof

After submission of this paper, a related report was published by Beck and Brudvig (1987). These authors also find that illumination at 200 K of PSII membranes pretreated with NH_2OH in the dark produces a new EPR signal from an unknown species between 3100 and 3400 G with $g = 2.1$. They provide a tentative assignment consistent with ours, suggesting a possible identity as an electron acceptor, owing to its appearance in illuminated, Tris-washed PSII membranes that have no functional Mn donor. Our results disagree on two points. First, they assign this to a free radical species and on this basis claim that the greatly smaller signal intensity compared to that of signal II indicates a negligible spin concentration per reaction center. Our results on the microwave power saturation of the $g = 2.1$ signal show that it cannot be associated with an isolated radical center, but rather exhibits efficient relaxation analogous to that observed for the FeQ_A^- signals at $g = 1.9$ and $g = 1.82$ (Figure 9). These are known to arise from coupled spins (Fe^{2+}, $S = 2$, and Q_A^-, $S = {}^1/_2$) present at one per PSII and having very weak EPR signals owing to the large energy dispersion of their transitions (Dismukes et al., 1984; Butler et al., 1984). Consequently, any comparison of the signal intensity for the $g = 2.1$ signal, restricted to the region of this turning point, with that for signal II will result in a severe underestimate of the spin concentration, for the same reason that this approach fails in estimating the spin concentration for the $g = 1.9$ and $g = 1.82$ signals. A second difference with the results by Beck and Brudvig is that they do not observe a loss of the $g = 1.9$ signal accompanying the formation of the $g = 2.1$ signal in NH_2OH-treated samples illuminated at 200 K (Figure 2). The reason for this difference may be related to two factors: the

shorter incubation times with NH_2OH (5 min) and higher NH_2OH concentrations (200 μM) used by these authors may not be optimum for expression of the reversible reaction which causes the EPR signals to convert. Excess NH_2OH consumes the exogenous quinone DCBQ by reduction, which is needed to oxidize FeQ_A^- prior to illumination (the $g = 1.9$ signal) in order to form the $g = 2.1$ signal upon illumination. As we have shown in Figure 8, if too much NH_2OH is used, the $g = 1.9$ signal is not converted to the $g = 2.1$ signal. These differences may also be partly due to the presence of 30% ethylene glycol as cryoprotectant in the samples of Beck and Brudvig compared to 0.2 M sucrose in our samples. These changes are already known to influence the structure of the Mn complex in the S_2 state (Zimmermann & Rutherford, 1986) and may also modify the acceptor side.

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Registry No. NH_2OH, 7803-49-8; Mn, 7439-96-5.

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