Photosynthesis Research (Biggins, J., Ed.) Vol. 1, pp 13–16, Martinus Nijhoff, Dordrecht, The Netherlands.
Westerhuis, W. H. J., Vos, M., Van Dorssen, R. J., van Grondelle, R., Amesz, J., & Niederman, R. A. (1987) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. 1, pp 29–32, Martinus Nijhoff, Dordrecht, The Netherlands.
Williams, J. C. (1986) Ph.D. Thesis, University of California, San Diego.
Wright, S. W., & Shearer, J. D. (1984) *J. Chromatogr. 294*, 281–295.
Yen, G. S. L., Cain, B. D., & Kaplan, S. (1984) *Biochim. Biophys. Acta 777*, 41–55.

Binding of Hydroxylamine to the Water-Oxidizing Complex and the Ferroquinone Electron Acceptor of Spinach Photosystem II†

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ABSTRACT: The reaction between spinach photosystem II (PSII) membranes and hydroxylamine has been investigated by equilibrium titrations and flash-induced reactions with electron paramagnetic resonance (EPR) spectroscopy to monitor the odd-electron species, O_2 evolution rate, and manganese binding. Two high-affinity sites for NH_2OH reaction have been characterized. Binding to the first site occurs within the water-oxidizing complex (WOC) and produces the well-known two flash shift in O_2 evolution. The usual two-electron shift in O_2 yield is accompanied by a parallel two-electron shift in the yield of the S_2 multiline EPR signal. This reaction occurs in two steps—an initial reversible reduction of manganese by two electrons at low concentrations ($\leq$5 NH_2OH/PSII) followed by, at higher concentrations, further reduction that is irreversible due to the release of 3 out of 4 Mn/PSII. The titration curve suggests that 2–3 Mn/PSII are released cooperatively, presumably from a common site. Binding to the second high-affinity site ($\leq$6 NH_2OH/PSII) produces a structural change in the ferrosemiquinone electron acceptor that is characterized by the conversion of the normal form of its EPR signal from $g = 1.9$ to a new form having $g = 2.1$. This structural change is blocked by herbicides, such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea, which block access to the Q_B acceptor site. The two flash delay in turnover seen at room temperature is lost at low temperatures (150–500 K) due to a block in multiple turnovers caused by NH_2OH. The site for the low-temperature blockage is undetermined but correlates with the structural change at the ferroquinone site. This suggests that the reoxidation of Q_A^- by Q_B following turnover is blocked, resulting instead in recombination upon warming. The reversible loss of both of the S_2-state EPR signals, the multiline and the $g = 4.1$ signals, caused by NH_2OH, titrated with identical curves, suggesting a common chemical reactivity and hence origin for these signals. The reaction between the S_2 state and NH_2OH occurs in less than 10 s and is considerably faster than binding to the (dark) S_1 state. The reversible binding of NH_2OH produces no stable paramagnetic products in the dark. The release of Mn by NH_2OH is followed by reduction of the oxidized donor D^+ responsible for EPR signal II_{slow} and signal II_{dark}, confirming earlier work establishing the accessibility of this donor to the aqueous phase through the Mn binding site [Ghanotakis, D. F., & Babcock, G. T. (1983) *FEBS Lett. 153*, 231–234].

Insight into the mechanism of O_2 evolution from the water-oxidizing complex (WOC) of photosynthetic membranes has come from studies of inhibitors which are substrate analogues such as NH_2OH (Bennoun & Joliot, 1969; Bouges, 1971; Cheniae & Martin, 1971).

There is considerable circumstantial evidence suggesting that NH_2OH binds to manganese within the active site of the complex [reviewed in Radmer and Cheniae (1977)]. Unlike substituted amines which inhibit O_2 evolution reversibly at high (millimolar) concentrations in direct proportion to their basicity (Ghanotakis et al., 1983), NH_2OH at comparable concentrations releases between two and four Mn(II) ions per photosystem II (PSII) (Cheniae & Martin, 1971; Yocum et al., 1981) and extrinsic proteins (Tamura & Cheniae, 1985), while being consumed at least partially to form N_2 (Radmer, 1983). In the mechanism proposed by Radmer (1983) a two-step process involving initial reduction of S_1 to S_0 occurs, followed by displacement of water bound to Mn by another molecule of NH_2OH. This suggests that reduction to the more labile Mn(II) oxidation state precedes the release of manganese. N- and O-substituted derivatives of NH_2OH were found to be less reactive, in a manner which correlated with their size rather than their redox potential (Radmer & Ollinger, 1983, 1984). The evidence which has accumulated to date has been unable to determine if the reduction of Mn occurs by an outer-sphere electron-transfer mechanism or by direct binding to Mn.

Preincubation with NH_2OH at low (micromolar) concentrations in the dark (S_1 state) results in the retardation of the flash-induced yield of O_2 by two electrons (Bouges, 1971). Similar results were found with H_2O_2 (Velthuys & Kok, 1978). Other phenomena coupled to electron transport such as PSII fluorescence emission (Theg et al., 1984; Itoh et al., 1984), proton release during short flashes (Foster & Junge, 1986a), and UV absorption transients attributed to Mn oxidation (Witt

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et al., 1986) are also retarded by two flashes. The system behaves as though it were reduced in the dark from the S_1 oxidation state to an effective oxidation state S_{-1} which is not formed during normal turnover (Bouges, 1971; Radmer & Ollinger, 1983). An alternative mechanism in which S_2 is reduced by NH_2OH rapidly following a flash would also be consistent.

The number of NH_2OH molecules involved in this reversible inhibition was found to be three to four on the basis of Hill plots of the parallel retardation of proton release (Forster & Jorge, 1986a,b). The dark equilibrium between NH_2OH and the WOC in thylakoid membranes is rather slow; a half-time of approximately 1 min for the S_1 state has been reported (Hanssum & Renger, 1985). Recently the reaction of NH_2OH and N_2H_4 with the dark S_1 state at 273 K has been monitored directly with electron paramagnetic resonance (EPR) to detect the yield of the S_2 multiline EPR signal for the manganese center formed by illumination at 200 K in spinach PSII membranes (Andreasson & Hansson, 1986). These authors found no reaction for ≤ 100 μM NH_2OH, while a slow reaction occurred above 100 μM NH_2OH ($t_{1/2} \gg 30$s) which was accompanied by irreversible release of Mn^{2+}. The rate of reaction between NH_2OH and the S_2 state at 273 K was followed, and the decay of the S_2 state was found to be accelerated by 50% to $t_{1/2} = 15$ s at 273 K. The fate of the reactive S_2 state was not determined in this study. A faster reactivity ($t_{1/2} \sim 1$ s) for N_2H_2 and NH_2OH with the S_2 and S_3 states compared to S_1 is also seen in flash O_2 yield studies on chloroplasts Hanssum & Renger, 1985). These studies also showed that the kinetics of reaction are complex (for NH_2OH). They were interpreted to involve sequential one-electron reduction steps, $S_2 \rightarrow S_1$ and $S_3 \rightarrow S_2 \rightarrow S_1$. Also, the S_1 state reached after two flashes is less susceptible to further NH_2OH deactivation compared to the S_1 state in uninhibited samples.

In addition to its reaction with the WOC, NH_2OH also interrupts electron transfer to the reaction center between Z and P_{680} at millimolar concentrations (De Haan et al., 1976; Ghanotakis & Babcock, 1983). The reactivity of spinach thylakoid membranes with NH_2OH in the dark and in the light has been reported to differ. Conflicting results claiming a faster release of Mn and a larger yield under illumination (Chenaie & Martin, 1971; Horton & Croze, 1977) or in the dark (Sharp & Yocum, 1981) have appeared.

We have investigated the mechanism of NH_2OH inhibition of the WOC in the dark by titration of a number of EPR-detectable donors to PSII, including the S_2 multiline EPR signal, the $g = 4.1$ signal, cytochrome *b*-559$^+$, and EPR signal II. These signals have been compared to the steady-state O_2 rate and to the extent of Mn release. The reactivity of NH_2OH under sequential turnover of the S states has also been examined by EPR of PSII membranes illuminated by either saturating flashing light at 277 K or by CW illumination at lower temperatures.

Materials and Methods

PSII membranes were prepared by the procedure described by Yamamoto (Yamamoto et al., 1981) or the modified BBY procedure (Berthold et al., 1981; Ford & Evans, 1983) and stored in a high sucrose buffer (400 mM) or in a 200 mM sucrose buffer with 30% glycerol at −80 °C. The O_2 rate activities ranged from 250 to 350 μM O_2·(mg of Chl)$^{-1}$·h^{-1} at pH 6.5 with a Clarke-type electrode at 20 °C. The electron acceptor used was 0.5 mM phenyl-*p*-benzoquinone (PPBQ) or 2,5-dichloro-*p*-benzoquinone (DCBQ) in methyl alcohol. The Q_B site inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), purchased from Sigma, was recrystallized prior to use. All the suspension buffers contained 200 mM sucrose, 20 mM MES (4-morpholineethanesulfonic acid), and 15 mM NaCl. PSII membranes were initially washed with 2 mM EDTA (ethylenediaminetetraacetic acid) in suspension buffer. The number of chlorophylls per photosystem II in the BBY-type spinach PSII membranes used here was taken as 225 per/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).

For treatment with NH_2OH the membranes were resuspended to a chlorophyll concentration of 1.2–2.0 mg/mL. The membranes were dark-adapted for at least 30 min at 273 K prior to incubation with NH_2OH in order to favor population of the S_1 state. NH_2OH was then added in the dark, the suspension was gently stirred for 30 min, and the membranes were pelleted at 48000*g* for 30 min. The supernatant and pellet were separated, and each was analyzed for manganese. The pellet was also assayed for O_2 activity and by EPR. For the flash experiments NH_2OH was added to the PSII membranes in the dark at a chlorophyll concentration of 1–1.5 mg/mL and incubated for 5 min in the dark, and then 5 mM $CaCl_2$ and the acceptor were added. The $CaCl_2$ was added for the flash experiments to give efficient S-state turnover. The samples were then directly transfered to EPR tubes, flashed, and immediately frozen. The total time between addition of NH_2OH and freezing was about 15 min.

Manganese was determined by atomic absorption (AA), utilizing a graphite furnace for sample atomization (Perkin-Elmer 305B). The supernatants were treated with 1% nitric acid before analysis. The resuspended pellets were vigorously homogenized in 10% nitric acid and centrifuged at 48000*g* for 30 min. The supernatant was analyzed for manganese, and the pellet was discarded after confirming the absence of manganese. This gave more reliable results than when manganese was not separated from the pellet.

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. Difference spectra were obtained with a Digital MINC-11 computer and a Lecroy WD8265 waveform digitizer or the A/D module of the Digital MINC-11 computer. The S_2 multiline EPR signal was observed at 10 K in the dark after illumination at 200 K by a tungsten source or after the samples were flashed at 277 K by an expanded laser beam from a Nd/YAG laser (Molectron MY-34) operating at 532 nm with a pulse duration of 15 ns and energy of 25 mJ/pulse. A representative spectrum of the S_2 multiline signal produced by low-temperature illumination is given in Figure 1A.

The $g = 4.1$ signal was generated by continuous illumination at 150 K and observed at 10 K as the difference spectrum of light − dark samples (Figure 1B). Cytochrome *b*-559 was monitored as the oxidized low-potential form by EPR at $g = 2.95$.

Results

Binding of NH_2OH to the S_1 State. The yield of the S_2-state EPR signals was used as an indicator of the extent of loss or blockage of the precursor state S_1. Curve 1 in Figure 2A shows a titration in the dark at 277 K of the yield of the S_2 multiline EPR signal against added NH_2OH. The S_2 signal was produced by illumination after freezing to 200 K. All data in Figure 2 are the average of three experiments. Curve 1 shows a linear decrease in the yield of the S_2 multiline EPR signal in direct proportion with NH_2OH. A 50% loss occurs at 2 NH_2OH/225 Chl, and an X intercept occurs at 5 NH_2OH/225 chlorophylls (one photosynthetic unit corre-

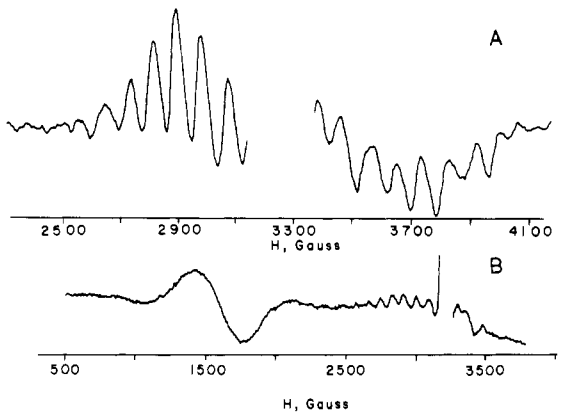

FIGURE 1: (A) EPR spectrum of spinach PSII membranes, illuminated at 200 K after dark adaptation for 30 min at 277 K. Chlorophyll concentration = 4.5 mg/mL; exogenous acceptor = 0.5 mM DCBQ. EPR conditions: microwave frequency, 9.22 GHz; microwave power, 15 mW; modulation amplitude, 32 G; sample temperature, 10 K. (B) Light minus dark EPR spectrum of spinach PSII membrane illuminated at 150 K after dark adaptation for 30 min at 277 K. EPR conditions same as for (A) except microwave power = 45 mW.

sponds to 225 Chl); 1 NH_2OH/225 Chl corresponds to 4–10 μM NH_2OH, depending on the chlorophyll concentration used during treatment. Evidence that this loss is photoreversible is given in curve 2. This shows the S_2 multiline EPR signal yield after consumption of NH_2OH by photooxidation. This is done by warming the treated samples to 4 °C, exposing them to continuous light from a 20-W tungsten source for 2 min, followed by a second dark adaptation for 30 min, and reillumination at 200 K. The difference between curves 1 and 2 indicates the fraction of centers which lose the multiline signal reversibly. Curve 3 shows that the steady-state O_2 evolution rate closely parallels the yield of centers which retain the photoreversible multiline signal in curve 2. Both curves go to zero above 1.5 mM NH_2OH. The difference between curve 2 and the control yield is attributed to centers which lose the S_2 multiline signal irreversibly by reaction with NH_2OH. The reversible loss of the S_2 multiline signal below 6 NH_2OH/PSII is not accompanied by a change in the yield of oxidized low-potential cytochrome *b*-559, as observed by its resonance at $g = 2.95$ (data not shown).

The photoreversible loss of the multiline signal could be attributable to the reaction of NH_2OH either with precursor S_1 state at 277 K or with the light-induced S_2 state at 200 K. The irreversible loss of the muliline signal at the higher NH_2OH concentrations seen in curve 2 of Figure 2A is accompanied by a parallel release of Mn from the membranes into the supernatant as seen in Figure 2B. Thus, S_1 reacts at higher NH_2OH concentrations by a process which releases Mn^{2+} into solution, in agreement with earlier findings (Cheniae & Martin, 1971). The photoreversible behavior is a new observation.

Having established that photoreversible reaction of NH_2OH can be observed in all centers at low concentrations without appreciable Mn release, we looked to see if the S_1 state, an EPR-silent state, reacts in the dark with NH_2OH to give an EPR-active state. This was done by obtaining difference spectra between dark untreated PSII and dark samples treated with 6 NH_2OH/PSII. The resulting difference spectra (5 K – 25 K) showed no changes (data not shown). This means either that no reaction occurs or that no stable EPR signals are formed.

Binding of NH_2OH Induces Loss of the $g = 4.1$ S_2-State EPR Signal. The $g = 4.1$ EPR signal was originally identified with an electron carrier between P680 and the water-oxidizing

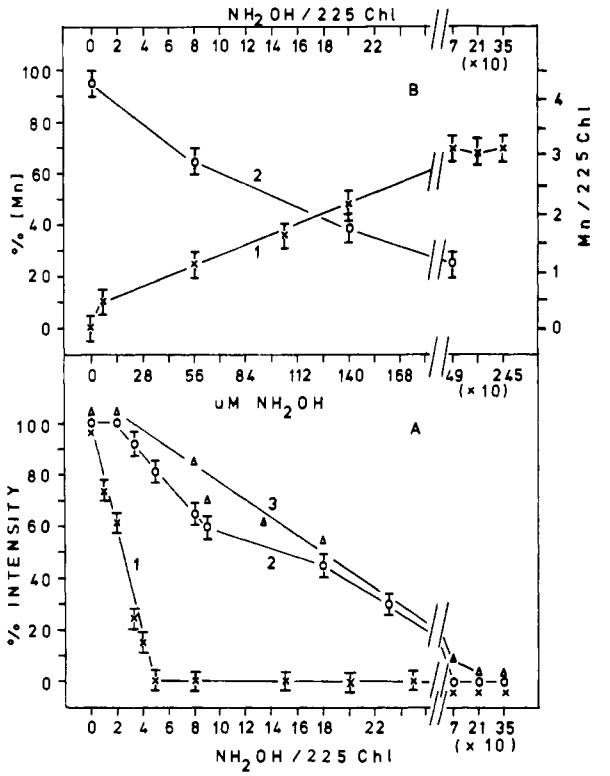

FIGURE 2: (A) Effect of NH_2OH on the O_2 rate and the yield of the S_2 multiline EPR signal of spinach PSII membranes. chlorophyll concentration = 6–8 mg/mL. Suspension buffer: 200 mM sucrose, 20 mM MES, and 15 mM NaCl at pH 6.0. Exogenous acceptor: 0.5 mM of either DCBQ or PPBQ. (Curve 1) Yield of the S_2 multiline EPR signal generated by CW illumination at 200 K after incubation with NH_2OH in the dark for 15 min followed by centrifugation and resuspension in buffer. (Curve 2) Yield of the S_2 multiline EPR signal produces by CW illumination at 200 K after consuming the NH_2OH from the samples in curve 1 by photooxidation at 277 K and re-dark adapating. (Curve 3) Steady-state O_2 rate after treatment (1). (B) Total Mn content of the supernatant and the depleted membrane after treatment (1). (Curve 1) Supernatant; (curve 2) NH_2OH-depleted membranes. EPR conditions: same as for Figure 1A.

complex (Casey & Sauer, 1984; Zimmerman & Rutherford, 1984). However, more recent studies have shown that it correlates with the S_2 state of the water-oxidizing complex (dePaula et al., 1985; Zimmerman & Rutherford, 1986). Two alternative models suggest that it may arise from an altered form of the same manganese cluster responsible for the multiline EPR signal (dePaula et al., 1986) or from a monomeric Mn(IV) center (Hansson et al., 1986). To test these ideas, a titration of this signal with NH_2OH was carried out as shown in curve 1 in Figure 3. These data are the average of two experiments. We see that about 50% of the signal is lost at 3 NH_2OH/225 Chl and 100% is lost at 5.5 NH_2OH/225 Chl. For the purpose of comparison with the S_2 multiline data of Figure 2A, curves 1 and 2 have been replotted on the expanded scale of Figure 3, curves 1′ and 2′. In curve 2 we see that recovery of the $g = 4.1$ signal occurs after consumption of the NH_2OH by photooxidation at 277 K. It is clear that the loss seen in curve 1 for the $g = 4.1$ signal is completely photoreversible and coincides with the S_2 multiline EPR signal shown as curves 1′ and 2′ (replotted from Figure 2A on the expanded scale in Figure 3). This shows that NH_2OH has essentially the same reactivity with the species giving rise to both the S_2 multiline EPR signal and the $g = 4.1$ signal. Small differences in these two data sets are probably due to the use of independent samples. This evidence supports the view that these two signals are monitoring alternative spectral forms of the same chemical species or

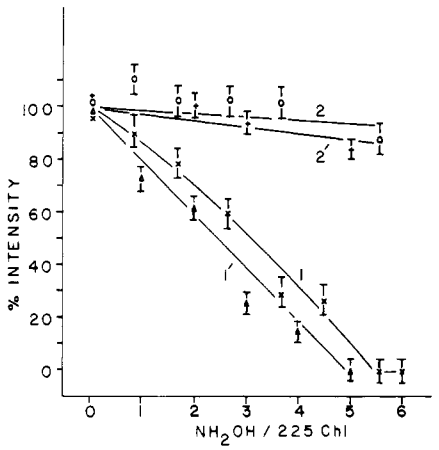

FIGURE 3: Effect of NH_2OH on the yield of the $g = 4.1$ signal generated by CW illumination at 150 K. Suspension buffer same as for Figure 2. Chlorophyll concentration = 6–8 mg/mL; exogenous acceptor = 0.5 mM of either DCBQ or PPBQ. (Curve 1) Yield of $g = 4.1$ after incubation in the darrk for 15 min, followed by centrifugation and resuspension. (Curve 2) Yield of $g = 4.1$ signal after consumption of NH_2OH by photooxidation at 227 K and dark readapting. EPR conditions same as for Figure 1B. (Curve 1') Data of Figure 2 (curve 1) redrawn on an expanded scale. (Curve 2') Data of Figure 2 (curve 2) redrawn on an expanded scale.

components that are in redox equilibrium. Andreasson and Hansson (1986) found that irreversible loss of the $g = 4.1$ signal occurs at higher [NH_2OH] and is accompanied by the irreversible loss of the S_2 multiline signal and the release of Mn. The reversible behavior at low [NH_2OH] has not been previously investigated.

Release of Manganese by NH_2OH. The total number of Mn atoms per PSII varied between 4.5 and 5 for different preparations. Curve 1 in Figure 2B shows the Mn released in the dark, while curve 2 shows the total Mn retained in the pellet as a function of NH_2OH concentration. The sum of the two curves is constant. A 100% loss of O_2 activity occurs at 1.5 mM NH_2OH and is accompanied by the release of about 75% Mn into the supernatant; the rest of the Mn is not extractable by NH_2OH alone and is retained in the pellet. Release of the initial 15% does not affect the O_2 activity, suggesting that this population may be extraneous Mn which is not removed by the initial wash with EDTA. Release of the next 27% results in the loss of 50% O_2 activity, and the release of the next 33% Mn results in complete loss of O_2 activity. These results are in agreement with earlier work (Tamura & Cheniae, 1985). From a comparison of panels A and B of Figure 2, there is a direct correspondence between irreversible loss of multiline signal, loss of O_2 rate, and the release of Mn. These data also indicate that the release of Mn by NH_2OH lags behind the reversible loss of the multiline signal. This indicates that the reaction between NH_2OH and manganese occurs in two distinct stages, an initial reversible reduction or blockage of the S_2-state signal followed by release of manganese at higher concentrations of hydroxylamine.

Reaction of NH_2OH with the Ferroquinone Acceptor. In searching for new EPR signals arising from the reaction products of hydroxylamine, a clear change was found in the $g = 1.9$ resonance associated with one of the semiquinone forms of the ferroquinone electron acceptor FeQ_A^- (the $g = 1.82$ is the other form). The assignment of these signals follows that given by Zimmerman and Rutherford (1984) and Rutherford and Zimmerman (1984), respectively. Figure 4A shows the light – dark difference EPR signal for control PSII membranes treated with 150 μM DCMU, illustrating the $g = 1.9$ and $g = 1.82$ signals for FeQ_A^-. The analogous difference spectrum for PSII membranes pretreated with 7 NH_2OH/PSII in the dark and without DCMU, Figure 4C, shows the absence of the $g = 1.9$ signal and the presence of a new signal at $g = 2.1$. Figure 4B shows that this apparent shift in g value can be blocked by first incubating the sample with 150 μM DCMU to block access into the Q^B site. This new signal is absent in the Mn-depleted samples obtained by treatment with excess NH_2OH. In its place the original $g = 1.9$ signal appears (data not shown). The $g = 2.1$ signal arises from a strongly relaxing paramagnetic center with a microwave saturation curve similar to the $g = 1.9$ signal (data not shown). On this basis we attribute this to a structurally altered form of the FeQ_A^- center produced by reaction of less than 7 NH_2OH with the ferroquinone acceptor. This will be the subject of a future study. Prior studies of the binding of the Q_B inhibitor atrazine have shown that 1 mM NH_2OH suppresses the binding of this inhibitor to PSII membranes (Renger et al., 1986).

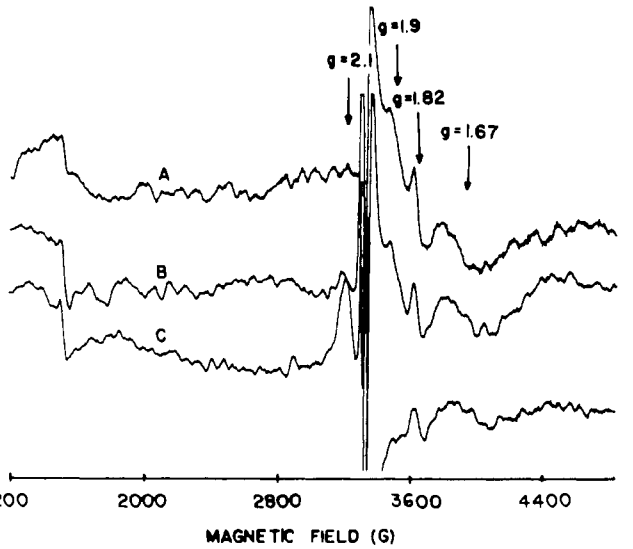

FIGURE 4: Effect on NH_2OH on the ferroquinone acceptor site of spinach PSII membranes. Chlorophyll concentration = 4.0 mg/mL. Suspension buffer same as for Figure 2. (Curve A) Light – dark difference EPR spectrum for control PSII membranes treated with 150 μM DCMU and illuminated at 200 K. (Curve B) Light – dark difference EPR spectrum for PSII membranes pretreated with 150 μM DCMU in the dark and then with 7 NH_2OH/PSII and illuminated at 200 K. (Curve C) Light – dark difference EPR spectrum for PSII membranes pretreated with 7 NH_2OH/PSII in the dark, and then 0.5 mM DCBQ was added and illuminated at 200 K. EPR conditions same as for Figure 1A except sample temperature = 5 K and microwave power = 40 mW.

Triple Turnover of PSII in the Presence of NH_2OH at 200 K. A triple turnover experiment was performed to test if three turnovers would be sufficient to reverse the two-flash deactivation induced by NH_2OH at low concentrations as seen by flash O_2 yield (Bouges, 1971). The first turnover was performed by illumination at 200 K, which normally causes transfer of one electron from the water-oxidizing complex to Q_A. The reason for not being able to transfer more than one electron is that the primary acceptor is reduced and its reoxidation by $Q_A^- \rightarrow Q_B$ electron transfer does not take place at 200 K, as has been revealed by thermoluminescence (Rutherford et al., 1982). However, a second electron may be transferred by first warming the sample in the dark above 250 K to reoxidize Q_A^- in the presence of an acceptor like DCBQ, similar to that shown by Rutherford et al (1982).

PSII samples were treated with 6.5 NH_2OH/225 Chl, and then 0.5 mM DCBQ was added before freezing in the dark. Figure 5 shows the yield of the S_2 multiline EPR signal in

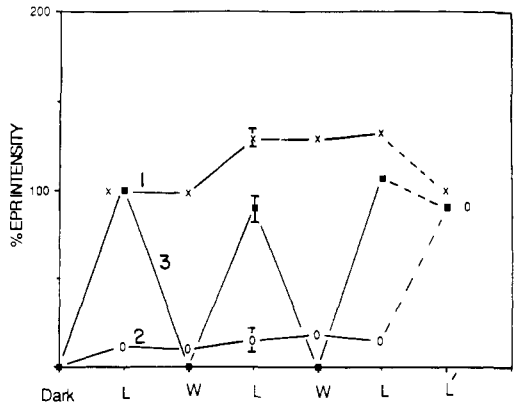

FIGURE 5: Triple turnover at low temperature of untreated and NH_2OH-treated (6.5 NH_2OH/255 Chl) PSII membranes. Suspension buffer same as for Figure 2. Chlorophyll concentration = 3–4 mg/mL; exogenous acceptor = 0.5 mM DCBQ. Curves 1 and 2 show the yield of the S_2 multiline EPR signal in control and NH_2OH-treated PSII membranes, respectively, upon successive illuminations at 200 K (L) and warming in the dark to 260 K for 2 min (W). L′ refers to 200 K illumination of the control and NH_2OH-treated samples after they were reactivated by warming to 277 K, CW illuminated for 2 min, dark-readapted for 30 min, and refrozen. Curve 3 shows the yield of the $g = 1.82$ FeQ_A^- EPR signal in the NH_2OH-treated sample relative to the yield in the control sample. The EPR conditions for observing the S_2 multiline EPR signal are the same as for Figure 1A. The $g = 1.82$ FeQ_A^- EPR signal was observed at a temperature of 5 K and power of 40 mW.

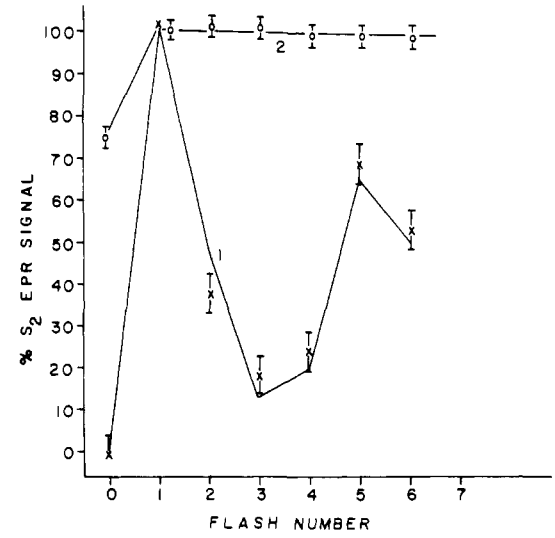

FIGURE 6: Yield of the S_2 multiline EPR signal (×) and signal II (○) as a function of laser flash number at 227 K. Chlorophyll concentration = 1–1.5 mg/mL. The PSII membranes contained 0.5 mM DCBQ and 5 mM $CaCl_2$ in addition to the suspension buffer of Figure 2. The membranes were frozen in a dry ice–methanol bath (200 K) and then in liquid N_2 immediately following the flash. (Curve 1) Predicted flash yield for an initital S-state distribution of S_0/S_1-(D)/$S_1/S_2/S_3$ = 0/0.25/0.75/0/0 with $\alpha = 0.1$ (misses) and $\beta = 0.05$ (double hits). EPR conditions are the same as for Figure 1A.

control (curve 1) and NH_2OH-treated samples (curve 2) upon alternate illumination at 200 K (L) and warming in the dark to 260 K for 2 min (W). The figure also shows the yield of the $g = 1.82$ FeQ_A^- EPR signal in the NH_2OH-treated sample, relative to the yield in control samples, during the illumination–warming cycles. We see that the second and third turnovers do not appreciably reverse the inhibition by NH_2OH; only a 5–10% increase is seen in the S_2 multiline EPR signal after the second and third turnovers. The fact that we are turning over the reaction center is evidenced by the oscillatory pattern of the $g = 1.82$ EPR signal (curve 3). Illumination at 200 K generates the $g = 1.82$ signal, and subsequent warming to 260 K completely eliminates the signal. If instead of warming the samples to 260 K in order to reoxidize FeQ_A^- we warm the samples to room temperature for 30 s prior to illumination at 200 K, we still do not see the appearance of the S_2 multiline EPR signal in the NH_2OH-treated samples after the second or third turnovers (data not shown). After three turnovers photooxidation of the NH_2OH by CW illumination at 277 K restores almost 100% of the S_2 multiline EPR signal (see L′). These results suggest either that more than three turnovers are required to eliminate the reversible NH_2OH effect or that NH_2OH interferes with low-temperature turnover of the S states while still permitting stable Q_A reduction. The former possibility is unlikely since in the next section we show that turnover at 277 K in the presence of NH_2OH effectively reduces the WOC by two electrons.

S_2-State Turnover in NH_2OH-Inhibited PSII at 277 K. Figure 6 shows the yield of the S_2 multiline EPR signal (curve 1) and signal II (curve 2) as a function of flash number in untreated (control) PSII membranes. These data are an average of two experiments. The predicted flash pattern for the S_2 multiline EPR signal given by curve 1 is calculated by assuming an initial S-state distribution of $S_0/S_1/S_1(D)/S_2/S_3$ = 0/0.75/0.25/0.0 with $\alpha = 0.1$ (misses) and $\beta = 0.05$ (double hits). The agreement with the experimental results is very good and thus provides a quantitative correlation between the S_2-state population and the multiline signal. These results are consistent with earlier qualitative estimates performed on more concentrated samples where light saturation was difficult to achieve (Dismukes & Siderer, 1981; Zimmerman & Rutherford, 1984).

The $S_1(D)$ state refers to those centers that have the manganese complex in the normal S_1 oxidation state along with the reduced state of donor D responsible for EPR signal II. Therefore, the "S_1" state is the only state populated in the dark, with 25% centers containing the reduced form of signal II. The reduced signal II is oxidized on the first flash and remains oxidized between flashes, giving an effective initial distribution with 25% centers in "S_0". This dark distribution of S states was observed previously to account for the relative yields of signal II and the S_2 multiline signal in PSII membranes (Damoder et al., 1986) and O_2 yield in chloroplasts (Vermaas et al., 1984; Velthuys & Visser, 1975). Formation of signal II by the S_2 state has been previously established (Babcock & Sauer, 1973). This assignment of 25% centers in the $S_1(D)$ state is also consistent with the observation that the yield of the S_2 multiline EPR signal produced by CW illumination at 200 K of membranes that were preilluminated with a single flash at 4 °C is about 25% greater than when membranes were just illuminated at 200 K or just single flashed at 4 °C (data not shown).

Curve 1 in Figure 7 shows the yield of the S_2 multiline EPR signal from PSII membranes treated in the dark with 7 NH_2OH/225 Chl. We see that maxima now occur on the third and seventh flashes instead of the first and fifth flashes, suggesting a two-electron reduction of the WOC. There is no change in the dark level of signal II vs control, and a single flash produces 90% of the control yield (data not shown). However, assuming an initial S-state distribution analogous to the control sample, except shifted by 2 equiv to a more reduced distribution, $S_{-1}(D)/S_{-1}/S_1/S_0/S_2/S_3$ = 0.25/0.75/0/0/0/0 with $\alpha = 0.1$ and $\beta = 0.05$, does not fit the data very well (curve 2). Here $S_{-1}(D)$ refers to centers with the WOC in an effective S_{-1} oxidation state with signal II reduced.

Reactivity of NH_2OH with the S_2 State. The S_2 state was generated quantitatively in PSII membranes at 1.5 mg of Chl/mL by a single intense laser flash, and then hydroxyl-

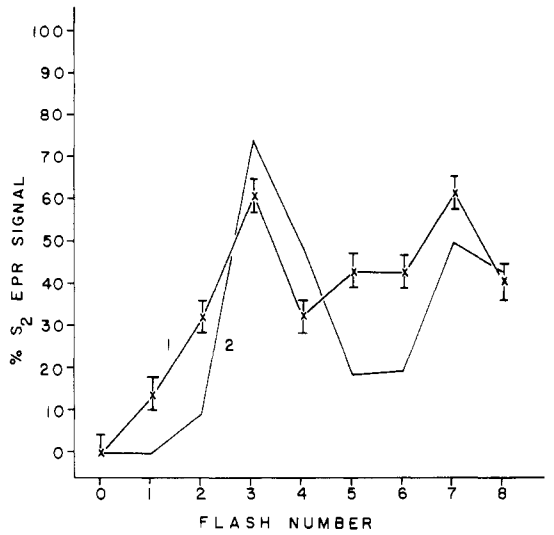

FIGURE 7: (Curve 1) Yield of the S_2 multiline EPR signal in dark-adapted PSII membranes incubated with 7 NH_2OH/225 Chl for 5 min in the dark followed by addition of 0.5 mM DCBQ and 5 mM $CaCl_2$ and flashed at 277 K and quench cooled to 200 K. Chlorophyll concentration = 1.0–1.5 mg/mL. (Curve 2) Predicted population of the S_2 state assuming a two-electron reduction of the WOC by NH_2OH. This is equivalent to an initial S-state population of S_{-1}-(D)/S_{-1}/S_0/S_1/S_2/S_3 = 0.25/0.75/0/0/0/0 with α = 0.1 (misses) and β = 0.05 (double hits). EPR conditions are the same as for Figure 1A.

amine (6 NH_2OH/PSII) was mixed with the sample and frozen immediately, the whole process taking less than 10 s. A control sample was treated identically except that buffer alone was added. No acceptor was used in this experiment, since the more effective acceptors like PPBQ and DCBQ react with NH_2OH. The yield of the S_2 multiline EPR signal generated by a single laser flash in control samples with no acceptor was about 40% of that in samples with an acceptor, while it was completely lost in the NH_2OH-treated samples. Subsequent 200 K illumination of the control gave 135% signal relative to the first single flash, and the NH_2OH-treated sample recovered only 10% of the control signal, indicating that NH_2OH has reduced the S_2 state by one or more electrons or that a block exists that cannot be overcome by illumination at 200 K. Since Q_A is reduced on the flash and is not oxidized in the absence of an acceptor on the time scale required for this experiment (10 s), the system is blocked on the acceptor side anyway, and further photooxidation is not expected. This experiment can only look at single turnover reactions. These results identify the S_2 state as a target for rapid reaction with NH_2OH (<10 s) even at low concentrations where the inhibition in reversible.

Reaction of NH_2OH with the Species Yielding EPR Signal II. Figure 8 shows the loss of the sum of the dark plus slow kinetic components of signal II as a function of NH_2OH concentration. This signal is completely lost at about 1.5 mM NH_2OH. Comparing Figures 2B and 6, we see that reduction of signal II_s by NH_2OH parallels the release of Mn.

DISCUSSION

Reversible Reactions of NH_2OH with S_1 and S_2. The X intercept of curve 1 in Figure 2A indicates that a maximum of 5 NH_2OH/PSII are required to reversibly and completely eliminate formation of the S_2 multiline signal. This is an upper limit to the number of NH_2OH molecules which react or bind at the manganese site in the dark (S_1) state, since we do not know the binding strength nor the number consumed by other reactions in the membrane.

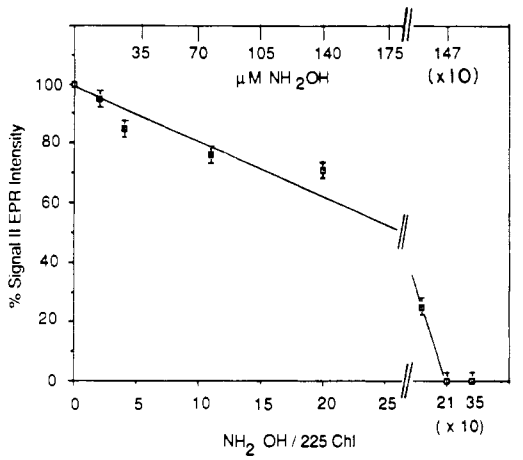

FIGURE 8: Effect of NH_2OH concentration on the yield of signal II in the dark. Chlorophyll concentration = 6–8 mg/mL; exogenous acceptor = 0.5 mM DCBQ. EPR conditions as in Figure 1 except microwave power = 0.5 mW and modulation amplitude = 4 G.

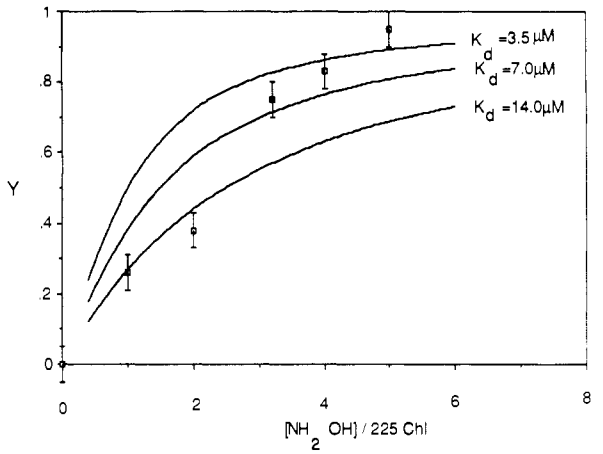

FIGURE 9: Analysis of the reversible loss of the S_2 multiline EPR signal by treatment with NH_2OH in the dark, in terms of a single binding site for NH_2OH at the WOC. Y is the fraction of WOC centers that have reacted with NH_2OH. The three solid lines are calculated assuming a single binding site characterized by dissociation constants (K_d) of 3.5, 7, and 14 μM, respectively, as shown. The data points are redrawn from Figure 2A, curve 1, with a small correction for the irreversible loss of O_2 activity at 5 NH_2OH/225 Chl.

The data of Figure 2A were compared to the calculated inhibition curves predicted by a model having a single binding site and a constant dissociation constant for NH_2OH binding:

$$\begin{array}{ccc} S_1 + NH_2OH & \overset{K_D^{-1}}{\rightleftharpoons} & S_1 \cdot NH_2OH \\ h\nu \downarrow & & \downarrow h\nu \\ S_2 & & X \end{array}$$

Figure 9 gives the results of this calculation for three dissociation constants (solid traces), and the experimental data are replotted from Figure 2A with a small correction to account for irreversible loss of O_2 evolution at 5 NH_2OH/PSII. In this plot the vertical axis Y is the fraction of centers blocked in S_2 formation by NH_2OH binding in S_1. This model does a poor job in predicting the experimental data. This could be interpreted two ways. Either the binding site is heterogeneous from one center to another, and so exhibits a distributed dissociation constant, or there are multiple sites for NH_2OH reaction. Evidence for the latter is seen in the reaction of NH_2OH with the ferroquinone acceptor (Figure 4). The former explanation does not agree well with the uniform behavior of PSII centers which is seen in the very normal S_2-state flash oscillation yield shown in Figure 6. Because NH_2OH

binds to the acceptor site in addition to the WOC, we are unable to analyze the titration curves to extract the binding constants at each site. Thus we can say only that a total of ≤ 5 NH_2OH/PSII bind to these sites. From measurements of the delay in proton release, Junge and Forster have deduced that three to four NH_2OH molecules bind cooperatively and reversibly at low concentration to the water-oxidizing complex (Forster & Junge, 1986a,b).

Recently Andreasson and Hansson have studied the reactivity of NH_2OH with the S_1 state in spinach PSII membranes (Andreasson & Hansson, 1986). In their experiments the loss of the S_2 multiline EPR signal generated by 200 K illumination of samples treated with NH_2OH in the dark does not show reversible behavior like that seen in curve 1 of Figure 2A. Rather, it follows the irreversible curve 2 of Figure 2A. Our results agree with theirs on the parallel release of Mn and loss of O_2 evolution (curve 2 of Figure 2B and curve 3 of Figure 2A, respectively). The most likely explanation of why they do not observe the reversible loss of the S_2 multiline signal at low NH_2OH is because their data are for samples that are washed to remove the NH_2OH prior to measurement. Although the binding of NH_2OH is sufficiently strong to preclude its release by washing (Diner & Joliot, 1977), inadvertant illumination during handling at such low concentrations would surely restore the uninhibited state.

Although our data establish that less than five NH_2OH molecules are needed for elimination of the S_2-state EPR signals, the titration data alone do not allow us to distinguish between the reduction of S_1 in the dark and reduction of S_2 upon illumination as the origin of the loss of the multiline signal.

The triple-turnover experiment conducted at 200 K (Figure 5) indicates either that NH_2OH reduces a majority of the centers by more than two electrons or that it interferes with the low-temperature formation of the normal manganese EPR signals—both the multiline and $g = 4.1$ EPR signals. Because reoxidation of Q_A^- occurred upon warming to 260 K in the presence of acceptor, the loss of multiline signal is not due to the lack of availability of oxidized Q_A centers. The flash experiment (Figures 6 and 7) which involves turnover at 277 K shows that NH_2OH effectively reduces the WOC by two electrons. Comparing the low-temperature turnover and the 277 K turnover experiments, it suggests that at low temperature NH_2OH also interferes with formation of the normal manganese EPR signals. This mode of inhibition by NH_2OH could be associated with the site responsible for the two-electron reduction, the ferroquinone site which produces the altered $g = 2.1$ signal (Figure 4), or the site between P680 and Z which blocks electron transfer (Den Haan et al., 1976; Ghanotakis & Babcock, 1983). Since the latter mode of inhibition has been reported only at considerably higher concentrations of NH_2OH (1–2 mM) than needed to inhibit low-temperature turnover in our experiments, we consider this mode unlikely. Thus, the site between P680 and Z is unlikely to be involved in this inhibition. On the other hand, binding of NH_2OH to the ferroquinone site might eliminate reoxidation of Q_A^- by Q_B, either by directly blocking the transfer or by reaction with Q_B. This would account for why the $g = 1.82$ EPR signal appears after each illumination at 200 K—Q_A photoreduction—and that recombination without net oxidation state advancement occurs upon warming (Figure 5). The identity of the donor responsible for Q_A reduction in NH_2OH-inhibited samples is not resolved by these data. Manganese oxidation is not ruled out, but this would require an EPR-silent form in both dark and illuminated samples.

The flash experiment in Figure 7 shows that the S_2 state exhibits the same two-flash retardation in the presence of hydroxylamine as does the O_2 yield (Bouges, 1971). There are several mechanisms that could account for this involving both two-electron reduction by hydroxylamine in the dark ($S_1 \rightarrow S_{-1}$) or after one flash ($S_2 \rightarrow S_0$) and successive one-electron reduction steps after the first two flashes ($S_1 \xrightarrow{h\nu} S_2 \rightarrow S_1 \xrightarrow{h\nu} S_2 \rightarrow S_1$). The data of Figure 7 do not fit very well with an initial S-state population distribution which assumes 100% of the S_1 and $S_1(D)$ centers are reduced in the dark by 2 equiv upon treatment with NH_2OH. The yield of the multiline signal on the first and second flash is too large for this assumption. There are two possible causes for this. The experimental flash data may be complicated by the incomplete binding of hydroxylamine to all centers and by weak background light used during transfer of samples to EPR tubes and during the flashing process. These factors appear to be responsible for the nonzero yield on the first flash (15%). The background light was not a problem for the titration experiments, since these samples contain much higher Chl concentrations, 6–8 mg/mL, as opposed to the 1–1.5 mg/mL needed for the flash experiments. However, these data clearly show that a majority of the centers undergo a two-electron shift induced by NH_2OH. This experiment gives no direct information on whether this occurs by reduction of S_1 in the dark or only after formation of S_2 following a flash.

Our results show that NH_2OH is able to reduce the S_2 state formed by a short flash at 277 K, completely within a few seconds at low concentrations. Andreasson and Hansson (1986) also found a faster reactivity between NH_2OH and the S_2 state. This was done by first generating the S_2 state at 273 K by CW illumination of PSII membranes (9 mg of Chl/mL) incubated with 50 μM DCMU and then adding 80 μM NH_2OH in the dark. The decay of the S_2 multiline EPR signal was twice as fast, about 15 s, as that in the control membranes. This compares with the much slower reaction with S_1 ($t_{1/2}$ = 1 min; Hanssum & Renger, 1985).

Reactivity of NH_2OH with Signal II. Signal II (both slow and dark kinetic forms) is completely reduced by much higher amounts of hydroxylamine (1.5 mM), and its reduction occurs only following the release of Mn. This suggests that accessibility to the site of signal II is controlled by Mn binding. This conclusion is in agreement with the enhanced decay kinetics observed earlier for the reduction of signal II by exogenous donors in Mn-free membranes (Yerkes & Babcock, 1980). An explanation for the high concentration of NH_2OH required to reduce signal II based on its redox potential seems unlikely, since signal II has a fairly high potential of 0.75 mV (Boussac & Etienne, 1984).

Irreversible Reaction of S_1 with NH_2OH. The complete irreversible loss of O_2 evolution at 1.5 mM NH_2OH (Figure 2A) is accompanied by the release of 70–80% of the total Mn (Figure 2B), with the initial 10% release being uncorrelated with the O_2 activity. Therefore, of the 4–5 Mn/PSII present initially, only 3.5–4.5 Mn/PSII need to be present for high O_2 evolution activity, in agreement with earlier work (Cheniae & Martin, 1971; Yocum et al., 1981). Release of the next 30% (1.2 Mn/PSII) results in the loss of 50% O_2 activity. Assuming that O_2 is evolved only from those centers that have an intact population of at least 4 Mn/PSII, this would mean that 2.4 Mn/PSII are released from 50% of the centers. This suggests that manganese is released cooperatively in amounts equal to about 2–3 Mn/PSII, rather than one per PSII. Release of the next 35% Mn (1.4 Mn/PSII) results in the complete loss of O_2 activity; i.e., a total of 2.7 Mn ions (68%)

are released from each PSII unit. The remaining 25% Mn (1.0 Mn/PSII) is not extracted by NH_2OH. There are two distinct pools of Mn with respect to NH_2OH extractability, one comprising a NH_2OH-labile pool of 3 Mn/PSII unit and the other 1 Mn/PSII which is either more sequestered or has a stronger binding affinity. Whether this is an indicator of two distinct Mn sites in the native WOC is not directly answerable by these results.

Tamura and Cheniae (1985) also studied the inactivation of O_2 evolution by NH_2OH and the solubilization of Mn and the extrinsic PSII polypeptides and found them to be closely correlated. Our results on the first two quantities and theirs are in agreement, although they find 0.5 Mn unextracted by NH_2OH treatment. When we compare their data on protein and Mn release, we note that the release of these by NH_2OH occurs only at considerably higher NH_2OH concentration than that which causes the reversible two-electron reduction of the S_2 state. The irreversible character at these higher concentrations might reasonably be attributed to Mn release triggered by more than a two-electron reduction of the WOC, possibly involving the other Mn ions that are not affected by the reversible reduction process at low concentration.

Mn(III) Oxidation of NH_2OH. Davis and Kustin (1969a,b) have extensively studied the reaction of Mn(III) with NH_2OH and NH_2NH_2 and their derivatives like *O*-methylhydroxylamine in homogeneous perchloric acid solutions at 25 °C. Their results were explained in terms of the following mechanism involving formation of the radical $NH_2O^\bullet$ in the rate-determining initital step (eq 1 or 2):

$$Mn^{3+} + NH_3OH^+ \rightarrow Mn^{2+} + NH_2O^\bullet + 2H^+ \quad (1)$$

$$MnOH^{2+} + NH_3OH^+ \rightarrow Mn^{2+} + NH_2O^\bullet + H_3O^+ \quad (2)$$

$$2H_2O + 5Mn^{3+} + NH_2O^\bullet \rightarrow 5Mn^{2+} + NO_3^- + 6H^+ \quad (3)$$

The major product of the reaction is NO_3^- in these strongly acidic solutions, rather than N_2 as found in PSII (Radmer, 1983; Radmer & Ollinger, 1983). The formation of nonvolatile products in the reaction with PSII has not been examined. The rate-limiting step is quite slow even though Mn^{3+} and $MnOH^{2+}$ are extremely potent oxidants. The rate constants are $k_1 = 1 \times 10^3\ M^{-1}\ s^{-1}$ and $k'_1 = 3 \times 10^3\ M^{-1}\ s^{-1}$, corresponding to pseudo-first-order lifetime of 100 s at $[Mn^{3+}] = 10^{-5}$ M in the initial reaction (eq 1). There is no evidence for formation of the paramagnetic species $NH_2O^\bullet$ in the reaction of PSII with NH_2OH. However, the stoichiometry of proton release of 1 H^+/NH_2OH in eq 1 or 2 is in agreement with the observed stoichiometry of proton release (Forster & Junge, 1986a,b) in the reaction of NH_2OH with the S_2 state of PSII: 2 H^+/N_2 = 2 H^+/2 NH_2OH. Here we have normalized the proton stoichiometry to N_2. This comparison suggests that a single reaction like eq 1 or 2 does not occur for Mn in the WOC but that a pair of these reactions on neighboring Mn sites could yield the observed H^+ stoichiometry with N_2 as the major product without formation of the $NH_2O^\bullet$ radical. The reaction of a simple dimanganese cluster in the S_1 or S_2 state could be considered as illustrative (eq 4 and 5). These equations are not intended to exclude higher

$$(S_2)WOC{\cdot}Mn_2^{III,IV} + 2NH_2OH \rightarrow (S_0)WOC{\cdot}Mn_2^{II,III} + N_2 + 2H_2O + 2H^+ \quad (4)$$

$$(S_1)WOC{\cdot}Mn_2^{III,III} + 2NH_2OH \rightarrow (S_{-1})WOC{\cdot}Mn_2^{II,II} + N_2 + 2H_2O + 2H^+ \quad (5)$$

nuclearity clusters of three or four Mn ions. The cumulative evidence favors reaction 4 over 5. Thus (1) there is no evidence yet reported for the release of protons or N_2 in the dark reaction of PSII with NH_2OH, while a single flash generates both protons (Forster & Junge, 1986) and N_2 (Radmer, 1983; Radmer & Ollinger, 1983) in NH_2OH-treated samples; (2) S_2 binds NH_2OH much faster than S_1; (3) X-ray absorption edge data support the reaction of S_2 but not S_1 with NH_2OH (Guiles et al., 1987).

On the basis of absorption changes in the UV attributed to charge accumulation by Mn photooxidation, Witt et al. (1986) proposed that S_1 reacts in the dark with NH_2OH to produce S_{-1} and that the source of proton release is attributable, at least in part, to the deprotonation of substrate H_2O. However, since they only report slow absorbance changes after flashing, they are unable to eliminate an alternative mechanism in which S_2 is the primary target for the photoreversible NH_2OH reduction. The preferential reactivity of the S_2 state with substrate analogues has also been seen with NH_3 (Beck et al., 1986). In this case, detectable binding requires about 100 mM NH_3, and reversible reaction with S_2 requires incubation near room temperature. In contrast, NH_2OH reacts with PSII at near stoichiometric levels, and this reaction occurs even at 150 K. This large difference suggests that the hydroxyl group is probably directly involved in the coordination of NH_2OH to Mn.

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Registry No. NH_2OH, 7803-49-8; Mn, 7439-96-5; O_2, 7782-44-7.

REFERENCES

Andreasson, L. E., & Hansson, O. (1986) in *Proceedings of the 7th International Congress on Photosynthesis* (Biggins, J., Ed.) Vol. I, pp 503–510, Martinus Nijhoff, Dordrecht, The Netherlands.

Babcock, G. T., & Sauer, K. (1973) *Biochim. Biophys. Acta 325*, 483–503.

Beck, W. F., dePaula, J. C., & Brudvig, G. W. (1986) *J. Am. Chem. Soc. 108*, 4018–4022.

Bennoun, P., & Joliot, A. (1969) *Biochim. Biophys. Acta 189*, 85–94.

Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231–234.

Bouges, B. (1971) *Biochim. Biophys. Acta 234*, 103–112.

Boussac, A., & Etienne, A. L. (1984) *Biochim. Biophys. Acta 766*, 576–581.

Cammarata, K., Tamura, N., Sayre, R., & Cheniae, G. (1984) in *Advances in Photosynthesis Research* (Sybesma, G., Ed.) Vol. I, pp 311–320, Martinus Nijhoff, Dordrecht, The Netherlands.

Casey, J. L., & Sauer, K. (1984) *Biochim. Biophys. Acta 767*, 21–28.

Cheniae, G. M., & Martin, I. F. (1971) *Plant Physiol. 47*, 568–575.

Damoder, R., Klimov, V. V., & Dismukes, G. C. (1986) *Biochim. Biophys. Acta 848*, 378–391.

Davis, G., & Kustin, K. (1969a) *Inorg. Chem. 8*, 484–490.

Davis, G., & Kustin, K. (1969b) *J. Phys. Chem. 73*, 2248–2253.

Den Haan, G. A., De Vries, H. A., & Duysens, L. N. M. (1976) *Biochim. Biophys. Acta 430*, 265–281.

dePaula, J. C., Innes, J. B., & Brudvig, G. W. (1985) *Biochemistry 24*, 8114–8120.

dePaula, J. C., Beck, W. F., & Brudvig, G. W. (1986) *J. Am. Chem. Soc. 108*, 4002–4009.

Diner, B. A., & Joliot, P. (1977) in *Encyclopedia of Plant*

Physiology (Avron, M., & Trebst, A., Eds.) pp 187–205, Springer-Verlag, Berlin.
Dismukes, G. C. (1986) *Photochem. Photobiol. 43*, 99–115.
Dismukes, G. C., & Siderer, Y. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 274–278.
Ford, R. C., & Evans, M. C. W. (1983) *FEBS Lett. 160*, 159–164.
Forster, V., & Junge, W. (1986a) *Febs. Lett. 186*, 153–157.
Forster, V., & Junge, W. (1986b) *Photosynth. Res. 9*, 197–210.
Ghanotakis, D. F., & Babcock, G. T. (1983) *FEBS Lett. 153*, 231–234.
Ghanotakis, D. F., O'Malley, P. J., Babcock, G. T., & Yocum, C. F. (1983) in *The O_2 Evolving System of Photosynthesis* (Inoue, Y., et al., Eds.) pp 91–101, Academic, Tokyo.
Ghanotakis, D. F., Babcock, G. T., & Yocum, C. F. (1986) *Biochim. Biophys. Acta 765*, 388–398.
Guiles, R. D., Yachandra, V. K., McDermott, A. E., Britt, R. D., Dexheimer, S. L., Sauer, K., & Klein, M. P. (1986) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. I, pp 561–564, Martinus Nijhoff, Dordrecht, The Netherlands.
Hansson, O., Aasa, R., & Vanngard, T. (1986) *Biophys. J. 51*, 825–832.
Hanssum, B., & Renger, G. (1985) *Biochim. Biphys. Acta 810*, 225–234.
Horton, P., & Croze, E. (1977) *Biochim. Biophys. Acta 462*, 86–101.
Itoh, S., Yerkes, C. T., Koike, H., Robinson, H. H., & Crofts, A. R. (1984) *Biochim. Biophys. Acta. 766*, 612–622.
Radmer, R. (1983) in *The O_2 Evolving System of Photosynthesis* (Inoue, Y., et al., Eds.) pp 135–144, Academic, Tokyo.
Radmer, R., & Cheniae, G. (1977) in *Primary Processes of Photosynthesis* (Barber, J., Ed.) Chapter 8, pp 303–348, Elsevier/North-Holland Biomedical, New York.
Radmer, R., & Ollinger, O. (1983) *FEBS Lett. 152*, 39–42.
Radmer, R., & Ollinger, O. (1984) in *Advances in Photosynthetic Research* (Sybesma, C., Ed.) Vol. I, pp 135–144, Martinus Nijhoff/Dr. W. Junk, The Hauge, The Netherlands.
Renger, G., Hagemann, R., & Fromme, R. (1986) *FEBS Lett. 203*, 210–214.
Rutherford, A. W., & Zimmermann, J.-L. (1984) *Biochim. Biophys. Acta 767*, 168–175.
Rutherford, A. W., Crofts, A. R., & Inoue, I. (1982) *Biochim. Biophys. Acta 682*, 457–465.
Sharp, R. R., & Yocum, C. F. (1981) *Biochim. Biophys. Acta 635*, 90–104.
Tamura, N., & Cheniae, G. (1985) *Biochim. Biophys. Acta 809*, 245–259.
Theg, S. M., Jursinic, P. A., & Homman, P. H. (1984) *Biochim. Biophys. Acta 766*, 636–646.
Velthuys, B. R., & Visser, J. W. M. (1975) *FEBS Lett. 55*, 109–112.
Velthuys, B. R., & Kok, B. (1978) *Biochim. Biophys. Acta 502*, 211–221.
Vermaas, W. F., Renger, G., & Dohnt, G. (1984) *Biochim. Biophys. Acta 764*, 194–202.
Witt, H. T., Saygin, O., Brettel, K., & Schlodder, E. (1986) in *Proceedings of the 7th International Congress on Photosynthesis* (Biggins, J., Ed.) Vol. I, pp 523–531, Martinus Nijhoff, Dordrecht, The Netherlands.
Yamamoto, Y., Doi, M., Tamura, N., & Nishimura, M. (1981) *FEBS Lett. 133*, 265–268.
Yerkes, C. T., & Babcock, G. T. (1980) *Biochim. Biophys. Acta 590*, 360–372.
Yocum, C. F., Yerkes, C. T., Blankenship, R. E., Sharp, R. R., & Babcock, G. T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7507–7511.
Zimmermann, J. L., & Rutherford, A. W. (1984) *Biochim. Biophys. Acta 767*, 160–167.
Zimmermann, J. L., & Rutherford, A. W. (1986) *Biochemistry 25*, 4609–4615.