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Reactions of Hydroxylamine with the Electron-Donor Side of Photosystem II†

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ABSTRACT: The reaction of hydroxylamine with the O_2-evolving center of photosystem II (PSII) in the S_1 state delays the advance of the H_2O-oxidation cycle by two charge separations. In this paper, we compare and contrast the reactions of hydroxylamine and *N*-methyl-substituted analogues with the electron-donor side of PSII in both O_2-evolving and inactivated [tris(hydroxymethyl)aminomethane- (Tris-) washed] spinach PSII membrane preparations. We have employed low-temperature electron paramagnetic resonance (EPR) spectroscopy in order to follow the oxidation state of the Mn complex in the O_2-evolving center and to detect radical oxidation products of hydroxylamine. When the reaction of hydroxylamine with the S_1 state in O_2-evolving membranes is allowed to proceed to completion, the S_2-state multiline EPR signal is suppressed until after three charge separations have occurred. Chemical removal of hydroxylamine from treated PSII membrane samples prior to illumination fails to reverse the effects of the dark reaction, which argues against an equilibrium coordination of hydroxylamine to a site in the O_2-evolving center. Instead, the results indicate that the Mn complex is reduced by two electrons by hydroxylamine, forming the S_{-1} state. An additional two-electron reduction of the Mn complex to a labile "S_{-3}" state probably occurs by a similar mechanism, accounting for the release of Mn(II) ions upon prolonged dark incubation of O_2-evolving membranes with high concentrations of hydroxylamine. In *N*,*N*-dimethylhydroxylamine-treated, Tris-washed PSII membranes, which lack O_2 evolution activity owing to loss of the Mn complex, a large yield of dimethyl nitroxide radical is produced immediately upon illumination at temperatures above 0 °C. The dimethyl nitroxide radical is not observed upon illumination under similar conditions in O_2-evolving PSII membranes, suggesting that one-electron photooxidations of hydroxylamine do not occur in centers that retain a functional Mn complex. We suggest that the flash-induced N_2 evolution observed in hydroxylamine-treated spinach thylakoid membrane preparations arises from recombination of hydroxylamine radicals formed in inactivated O_2-evolving centers.

The mechanism of photosynthetic O_2 evolution involves the catalysis of the four-electron oxidation of H_2O by a polynuclear Mn complex in the O_2-evolving center of photosystem II (PSII)[1] [for a recent review, see Babcock (1987)]. Electron paramagnetic resonance (EPR) spectroscopy (Dismukes & Siderer, 1981; Zimmermann & Rutherford, 1984) and X-ray absorption experiments (Goodin et al., 1984) have shown that Mn oxidation state changes occur as the O_2-evolving center advances through its five oxidation states S_i, i = 0–4, suggesting that the Mn complex functions to store the required oxidizing equivalents for the H_2O-oxidation reaction. The Mn complex appears to consist of four exchange-coupled Mn ions (Dismukes et al., 1982; de Paula et al., 1986a) that are proposed to be arranged in the S_2 state in a Mn_4O_4 cubane-like configuration (Brudvig & Crabtree, 1986).

Considerable effort has been expended in the past few years toward understanding the inhibition of photosynthetic O_2 evolution by primary amines, which might coordinate to Mn in the O_2-evolving center (Sandusky & Yocum, 1983, 1984, 1986). When the S_2-state multiline EPR signal was used as a probe for ligand-substitution reactions at the Mn complex,

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[1] Abbreviations: DCBQ, 2,5-dichloro-*p*-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; kDa, kilodalton; MES, 2-(*N*-morpholino)ethanesulfonic acid; PSII, photosystem II; P680, primary electron donor in PSII; Q_A, primary electron acceptor in PSII; Tris, tris(hydroxymethyl)aminomethane.

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NH_3 was observed to bind to the Mn complex only after formation of the S_2 state at temperatures high enough to allow facile ligand exchange. Under similar conditions, however, amines larger than NH_3 were unable to coordinate to the Mn complex (Beck et al., 1986; Beck & Brudvig, 1986). This specificity for small Lewis bases analogous to H_2O supports the proposal that the Mn complex forms the substrate H_2O-binding site of the O_2-evolving center.

Hydroxylamine, hydrazine, and hydrogen peroxide form a second class of small molecules that have been proposed to bind to the Mn complex [reviewed by Babcock (1987)]. While primary amines inhibit photosynthetic O_2 evolution (Sandusky & Yocum, 1983, 1984, 1986), molecules isoelectronic with hydroxylamine only delay the O_2-evolving cycle by 2 quanta after a relatively slow reaction with the Mn complex in the dark (Bouges, 1971; Kok & Velthuys, 1977; Velthuys & Kok, 1978a,b). A two-flash delay also occurs in the release of protons by the O_2-evolving center in hydroxylamine- or hydrazine-treated thylakoid membranes (Förster, 1984; Förster & Junge, 1985a–c, 1986). After treatment of PSII membranes with hydroxylamine in the dark, formation of the S_2-state multiline EPR signal by low-temperature illumination is suppressed (Casey & Sauer, 1984). Because hydroxylamine can react with the dark-stable S_1 state, whereas NH_3 binds to the Mn complex only after the S_2 state is formed, Babcock (1987) has pointed out that the interactions of NH_3 and of hydroxylamine with the O_2-evolving center are inconsistent with both molecules acting as substrate analogues. In an attempt to resolve this paradox, we recently showed that hydroxylamine does not bind in the dark as a substrate analogue since the rate of its reaction with the O_2-evolving center in the S_1 state depends inversely on the Cl^- concentration (Beck & Brudvig, 1987a). Primary amines also bind to the O_2-evolving center in the S_1 state in a manner inversely dependent on the Cl^- concentration (Beck & Brudvig, 1986, 1987b). Furthermore, N-alkyl substitutions decrease both the extent of binding of primary amines to the S_1 state and the rate of reaction of hydroxylamine with the S_1 state (Beck & Brudvig, 1986, 1987a,b). These results indicate that primary amines and hydroxylamines bind to the same site in the O_2-evolving center in the S_1 state, and this site appears to be the site at which Cl^- functions as a cofactor for the H_2O-oxidation reaction. It is evident that the reaction or binding of hydroxylamine to the Cl^--binding site affects the redox state of the Mn complex, perhaps through an electron-transfer reaction in the dark or immediately upon illumination.

The observation by Bouges (1971) that a maximal O_2 yield is obtained in hydroxylamine-treated *Chlorella* after five flashes of light, rather than the usual three required in untreated cells, suggested two types of models for the interaction of hydroxylamine with the O_2-evolving center (Bouges, 1971; Bennoun & Bouges, 1972; Bouges-Bocquet, 1973). One model, shown in eq 1–3, involves the reduction of the Mn

$$NH_2OH + S_1 \xrightarrow{\text{dark}} S_0 + \text{product} \tag{1}$$

$$NH_2OH + S_0 \underset{}{\overset{\text{dark}}{\rightleftharpoons}} S_0(NH_2OH) \tag{2}$$

$$S_0(NH_2OH) \xrightarrow{h\nu} S_0 + \text{product} \tag{3}$$

complex from the S_1 state to the S_0 state by a single hydroxylamine molecule, the equilibrium binding of a second hydroxylamine molecule to the S_0 state, and the subsequent oxidation of the bound hydroxylamine species during the first flash of light, producing the S_0 state. Subsequent flashes would cause the normal advance of the O_2-evolving center through the S states, eventually yielding the evolution of an O_2 molecule on the fifth flash. The initial reaction product of eq 1 and eq 3 would be a hydroxylamine (nitroxide) radical, although the hydroxylamine-derived product(s) has (have) not been identified.

A model similar to that discussed above has been used by Radmer and Ollinger (1982, 1983) to account for the evolution of N_2 on the first flash by thylakoid membranes treated with hydroxylamine. N_2 could be formed by recombination of hydroxylamine radicals formed following the first flash of light (Radmer, 1979). Radmer and Ollinger (1983) proposed that hydroxylamine molecules coordinate to the Mn complex as bidentate ligands prior to being oxidized. On the basis of the relative effects of alkyl-substituted hydroxylamines on the N_2 and O_2 yields measured in treated thylakoid membranes, Radmer and Ollinger (1983) formulated a topological model for the H_2O-binding site of the O_2-evolving center. Owing to the observed dependence of the rate of hydroxylamine's reaction in the S_1 state on the Cl^- concentration (Beck & Brudvig, 1987a), the model of Radmer and Ollinger (1983) is unlikely to apply to the substrate-binding site; however, it may pertain to the shape of the Cl^--binding site.

A second model, preferred by Bouges-Bocquet (1973) on the basis of flash O_2-evolution experiments, involves the equilibrium coordination of two molecules of hydroxylamine to the S_1 state. Two sequential flashes of light are suggested to cause the oxidation of the bound hydroxylamine molecules, leaving the O_2-evolving center in the S_1 state.

$$2NH_2OH + S_1 \overset{\text{dark}}{\rightleftharpoons} S_1(NH_2OH)_2 \tag{4}$$

$$S_1(NH_2OH)_2 \xrightarrow{h\nu} S_1(NH_2OH) + \text{product} \xrightarrow{h\nu} S_1 + \text{product} \tag{5}$$

Again, the initial reaction product in eq 5 is a hydroxylamine radical. A derivative of this model was proposed by Förster (1984), in which an equilibrium binding of two hydroxylamine molecules in the S_1 state, as in eq 4, is followed by the two-electron reduction of the O_2-evolving center following the initial flash of light:

$$S_1(NH_2OH)_2 \xrightarrow{h\nu} S_0 + \text{product} \tag{6}$$

Andréasson and Hansson (1987) have examined the effect of hydroxylamine on the rate of the $S_2 \rightarrow S_1$ back-reaction by following the intensity of the S_2-state multiline EPR signal as a function of dark incubation time in the presence of hydroxylamine at 0 °C. In their experiment, the S_2 state was generated in spinach PSII membrane samples by illumination at 273 K; hydroxylamine was then immediately added to start the reaction with the S_2 state. It was observed that hydroxylamine enhanced the rate of decay of the S_2-state multiline EPR signal; a subsequent illumination at 200 K after the reaction did not recover a full yield of the S_2-state multiline EPR signal. This experiment provides the first evidence that the S_2 state can react with hydroxylamine. Andréasson and Hansson (1987) interpreted their results as showing that hydroxylamine reacts with the S_2 state through two paths. One path involves an enhancement of the normal charge-recombination reaction involved in the one-electron $S_2 \rightarrow S_1$ back-reaction; this reaction was thought to be similar to that involved in the interaction of certain lipophilic anions (Renger, 1972) with the O_2-evolving center. Andréasson and Hansson (1987) proposed that the other path involved a reduction of the O_2-evolving center by hydroxylamine, thus explaining the reduced yield of the S_2-state multiline EPR signal on the second illumination.

In this paper, we have employed low-temperature EPR methods in order to study the reaction of hydroxylamines with the O_2-evolving center in the dark-stable S_1 state. We previously demonstrated that hydroxylamine reacts to completion with the O_2-evolving center in the S_1 state with first-order kinetics with respect to the hydroxylamine concentration (Beck & Brudvig, 1987a). By studying the reaction of hydroxylamine with the S_1 state, rather than the S_2 state, we avoid the complication of a competition between the reaction of hydroxylamine in the S_2 state and the $S_2 \rightarrow S_1$ back-reaction. The three models discussed above for the nature of the interaction of hydroxylamine with the O_2-evolving center are tested by determining the effects of hydroxylamine, *N*-methylhydroxylamine, or *N,N*-dimethylhydroxylamine on the S state present before and after illumination of dark-treated spinach PSII membranes. The paths of electron donation in hydroxylamine-treated PSII membranes are determined by using the methods developed by de Paula et al. (1985). Further, we use EPR spectroscopy to check for the production of hydroxylamine (nitroxide) radicals during illumination in the presence of hydroxylamine in both O_2-evolving and Tris-washed PSII membranes. Our results suggest that hydroxylamine reduces the Mn complex in the dark by two electrons to an S_{-1} oxidation state, probably through an outer-sphere electron-transfer reaction, and that previously detected N_2 products of light-induced reactions, arising from recombination of hydroxylamine radicals, can be attributed to inactivated centers.

EXPERIMENTAL PROCEDURES

Materials. Hydroxylamine hydrochloride was used as received from Eastman Kodak. *N*-Methyl- and *N,N*-dimethylhydroxylamine hydrochloride were used as received from Aldrich. 2,5-Dichloro-*p*-benzoquinone (DCBQ), from Eastman Kodak, was recrystallized twice from 95% ethanol, and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), obtained from Sigma, was recrystallized 4 times from 95% ethanol before use. DCBQ and DCMU solutions were prepared in 95% ethanol immediately prior to use. 2-(*N*-Morpholino)ethanesulfonic acid (MES) and *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) were used as received from Research Organics.

Preparation of PSII Membrane Samples. O_2-evolving PSII membranes were isolated from market spinach leaves according to a Triton X-100 extraction procedure (Berthold et al., 1981), modified as previously described (Beck et al., 1985). O_2-evolving PSII membranes were stored at 77 K in a buffer solution at pH 6.0 containing 20 mM MES–NaOH, 15 mM NaCl, and 30% (v/v) ethylene glycol added as a cryoprotectant. This buffer solution was also used as the suspension medium for EPR samples. All steps in the isolation procedure were conducted in darkness, as described previously (Beck et al., 1985).

Tris-washed PSII membranes were made from thawed, dilute (<0.5 mg of chlorophyll/mL) suspensions of O_2-evolving PSII membranes treated with a 0.8 M Tris solution at pH 8.5, according to the procedure of Yocum et al. (1981). Tris-washed PSII membranes were resuspended and centrifuged 3 times in a buffer solution at pH 6.0 containing 20 mM MES–NaOH, 15 mM NaCl, 5 mM EDTA, and 30% (v/v) ethylene glycol, followed by two resuspension and centrifugation cycles in the suspension buffer solution described above. EDTA was present in order to chelate Mn(II) ions released from the O_2-evolving center.

EPR Spectroscopy. EPR samples of O_2-evolving PSII membranes were made from dark-adapted, resting-state preparations, as previously described (Beck et al., 1986). Stored PSII membrane suspensions (about 8–10 mg of chlorophyll/mL) at pH 6.0 were thawed in darkness for about 30 min, on ice, before additional handling. As required, the hydroxylamines were added to the PSII membrane suspension as solutions of the hydrochloride in the sample buffer solution. The final chlorophyll concentration was between 4 and 5 mg of chlorophyll/mL. Hydroxylamine-treated samples were incubated on ice for the indicated period before being frozen in liquid N_2. All of the steps used in preparing the EPR samples were conducted in darkness. EPR samples of 10-min dark-adapted, Tris-washed PSII membranes were prepared in an identical fashion.

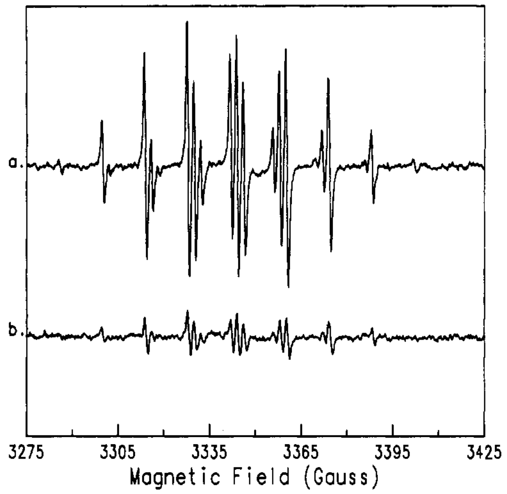


FIGURE 1: Dimethyl nitroxide radical EPR signals observed in *N,N*-dimethylhydroxylamine-treated, Tris-washed PSII membranes at pH 6.0. Tris-washed PSII membranes (4.5 mg of chlorophyll/mL) were treated with 5 mM *N,N*-dimethylhydroxylamine and were held in a capillary cell at room temperature (ca. 22 °C). Spectra were recorded with the same sample (a) under continuous illumination from a H_2O-filtered, quartz-halogen lamp and (b) in complete darkness, 15 min following illumination. EPR spectrometer conditions: microwave frequency, 9.5 GHz; microwave power, 600 μW; field modulation frequency, 100 kHz; field modulation amplitude, 1.25 G. Each spectrum is the average of four scans.

Sample illuminations were carried out as previously described (Beck et al., 1986). EPR spectra were obtained either at liquid He temperatures with the JEOL ME-3X spectrometer and associated instrumentation employed previously (Beck et al., 1985) or at liquid N_2 temperatures with a Varian E-9 spectrometer equipped with an E-101 microwave bridge and DEC LSI 11/23 computer. Sample temperatures were controlled either by an Oxford Instruments ESR-900 liquid He cryostat or by a Lake Shore Cryotonics DRC-84C temperature controller equipped with a N_2 flow cryostat. Difference spectra were obtained through computer subtraction of the dark background spectrum from the postillumination spectrum obtained under the same measurement conditions.

RESULTS

Electron Donation in Tris-Washed PSII Membranes in the Presence of Hydroxylamine. Tris-washed PSII membranes lack a functional Mn complex [for a review, see Amesz (1983)]. As a result, exogenous electron donors are photooxidized during illumination through one-electron reactions (Yerkes & Babcock, 1980). In the presence of *N,N*-dimethylhydroxylamine, Tris-washed PSII membranes produce a radical EPR signal when illuminated at room temperature (Figure 1a). We can assign this EPR signal to the dimethyl nitroxide radical by comparison with a previously published spectrum (Bielski & Gebicki, 1967). Figure 1b shows that

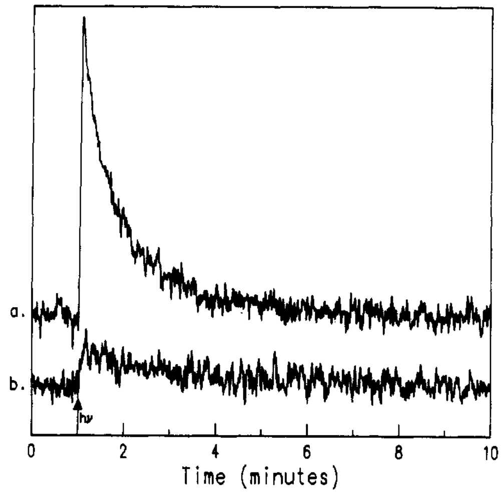


FIGURE 2: Time course for the production and decay of the dimethyl nitroxide radical in (a) Tris-washed and (b) untreated PSII membranes treated in darkness with 5 mM *N*,*N*-dimethylhydroxylamine. Samples were held in a capillary cell at room temperature (ca. 22 °C) at 4 mg of chlorophyll/mL for the Tris-washed PSII membranes and at 2.5 mg of chlorophyll/mL for the untreated PSII membranes. The samples were illuminated for 2 s at the arrow; the conclusion of the trace was acquired in complete darkness. EPR spectrometer conditions were as in Figure 1 except that the magnetic field was fixed at 3327 G. The output time constant for the phase-sensitive detector was set to 1.0 s.

a small, steady-state population of the dimethyl nitroxide radical persists in the sample in the dark, well after illumination. It can be concluded that autoxidation of *N*,*N*-dimethylhydroxylamine occurs to some extent under our sample conditions. A similar intensity of the dimethyl nitroxide radical EPR signal, as in Figure 1b, is observed in a sample not containing PSII membranes (data not shown).

Figure 2a shows the production and subsequent decay in darkness of the dimethyl nitroxide radical EPR signal. A 2-s illumination produces a large yield of the nitroxide radical signal in a Tris-washed PSII membrane sample (Figure 2a) within the time constant of the experiment, while only a small change in EPR signal intensity with a much slower onset occurs in an O_2-evolving PSII membrane sample (Figure 2b). A lower chlorophyll concentration was employed in the O_2-evolving PSII membrane sample in order to improve the degree of light saturation, but similar results were obtained at higher chlorophyll concentrations. The small intensity change observed in Figure 2b can be mostly attributed to an illumination artifact and can also be seen as a slowly decaying signal in Figure 1a. Thus, under these conditions, only Tris-washed PSII membrane samples produce a significant amount of dimethyl nitroxide when illuminated in the presence of *N*,*N*-dimethylhydroxylamine.

The signal in Figure 2a decays exponentially following the illumination period. Under our sample conditions at approximately 22 °C, the dimethyl nitroxide radical has a half-time for decay of approximately 1 min. The relatively long lifetime of this radical is due to the dimethyl substitution (Roberts, 1979). We performed similar experiments (not shown) with Tris-washed PSII membranes treated with either hydroxylamine or *N*-methylhydroxylamine. In these cases, we did not detect nitroxide radicals in either illuminated or dark samples. Since the expected nitroxide radicals are not fully *N*,*N*-dialkyl-substituted, their lifetimes are expected to be short enough at room temperature to preclude production of EPR-observable concentrations.

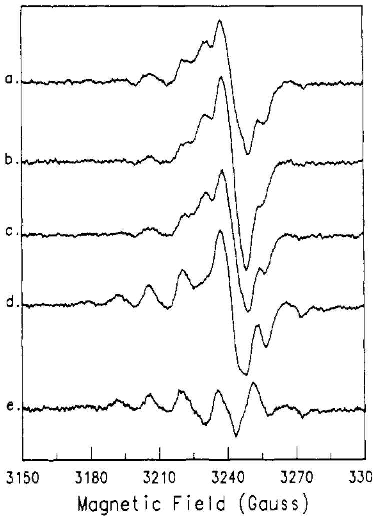


FIGURE 3: Radical EPR signals observed in *N*,*N*-dimethylhydroxylamine-treated, Tris-washed PSII membranes at pH 6.0. Tris-washed PSII membranes (4.8 mg of chlorophyll/mL) were treated with 25 mM *N*,*N*-dimethylhydroxylamine and with 100 μM DCMU: (a) dark sample; (b) after illumination at 210 K for 2 min; (c) the same sample was then incubated at −5 °C for 3 min; (d) following an additional dark incubation at 0 °C for 5 min, the sample was illuminated at 0 °C for 1 min; (e) difference spectrum, [(d) − (c)], at twice the relative gain used in the other spectra. EPR spectrometer conditions: microwave power, 200 μW; microwave frequency, 9.17 GHz; field modulation frequency, 100 kHz; field modulation amplitude, 4 G; sample temperature 100 K. Each spectrum is the average of two scans.

At lower temperatures, it might be possible to trap unstable nitroxide radicals formed after illumination of Tris-washed PSII membranes treated with hydroxylamines. As shown in Figure 3, however, the nitroxide radical is not produced after illumination at 210 K. Figure 3a shows an EPR spectrum from a dark, *N*,*N*-dimethylhydroxylamine-treated, Tris-washed PSII membrane sample. EPR signal II$_s$, attributed to a tyrosine cation radical species (Barry & Babcock, 1987), is superimposed upon the wider powder pattern of the small amount of dimethyl nitroxide radical present in dark samples. Following illumination at 210 K, the spectrum is changed only by the appearance of a narrow, 10-G wide radical EPR signal, which can be assigned to a chlorophyll cation radical (Visser & Rijgersberg, 1975). This radical EPR signal was previously observed in PSII membrane samples depleted of the 17- and 23-kDa extrinsic polypeptides (de Paula et al., 1986b). The chlorophyll cation is formed by illumination in Tris-washed PSII membranes because the other endogenous electron donors are either removed or already oxidized; cytochrome b_{559} is in the low-potential configuration (Erixon et al., 1972), owing to the loss of the 17- and 23-kDa extrinsic polypeptides during the Tris treatment, and is autoxidized in the dark (de Paula et al., 1986b). Note that the intensity of the dimethyl nitroxide radical EPR signal is the same as that observed in the dark spectrum. Thus, at this illumination temperature, the rate of electron transfer to P680$^+$ from exogenous *N*,*N*-dimethylhydroxylamine is much smaller than the rate of electron transfer from endogenous chlorophyll, despite the relatively high *N*,*N*-dimethylhydroxylamine concentration.

Figure 3c shows the result of warming the illuminated, *N*,*N*-dimethylhydroxylamine-treated, Tris-washed PSII membrane sample to −5 °C in darkness. The chlorophyll cation radical EPR signal decays quantitatively within 3 min at this temperature, but no change occurs in the intensity of the dimethyl nitroxide radical EPR signal. Figure 3d shows, however, that the dimethyl nitroxide radical can be trapped at low temperature in a Tris-washed PSII membrane sample by a brief illumination at 0 °C followed by freezing in liquid

N_2. The yield of the dimethyl nitroxide radical, displayed more clearly at higher gain in the difference spectrum shown in Figure 3e, is that produced by a single charge separation in the PSII reaction center since DCMU was present.

The results presented in Figure 3e show that the dimethyl nitroxide radical is produced in *N,N*-dimethylhydroxylamine-treated, Tris-washed PSII membranes only at temperatures approaching physiological conditions, where diffusional processes become more facile. Further, the mechanism of the dimethyl nitroxide radical formation involves a one-electron oxidation reaction mediated by electron donation to $P680^+$, probably through the intermediate Z^+ (Yerkes & Babcock, 1980), and does not proceed via other chlorophyll cation radicals or cytochrome b_{559}. We performed similar experiments (not shown) with Tris-washed PSII membranes treated with hydroxylamine or *N*-methylhydroxylamine; in both cases, illumination at 210 K produces only the chlorophyll cation radical EPR signal. The yield of the chlorophyll cation radical following illumination at 210 K is constant, even in the presence of high concentrations of hydroxylamine.

We were unable to trap EPR-detectable quantities of the nitroxide radical species following illumination at room temperature and rapid freezing of hydroxylamine- or *N*-methylhydroxylamine-treated, Tris-washed PSII membranes, no doubt owing to the short lifetimes of the radicals. The instability of nitroxide radicals without *N,N*-dialkyl substitution is noted by Roberts (1979). However, convincing evidence exists for the light-driven production of one-electron oxidized hydroxylamine species in Tris-washed PSII membranes treated with either hydroxylamine or *N*-methylhydroxylamine (Radmer et al., 1986). Nitroxide radicals are probably the source of the light-induced, amperometric signals observed by Bennoun and Joliot (1969) in spinach thylakoid membranes in the presence of high concentrations of hydroxylamine; at high hydroxylamine concentrations, Cheniae and Martin (1971) found that functional Mn is depleted from the O_2-evolving center, producing inactivated centers similar to those produced by treatment with Tris. Additionally, the flash-induced N_2 yields observed by Radmer et al. (1986) from hydroxylamine-treated, Tris-treated PSII membranes most likely arise from the recombination of nitroxide radicals produced by the one-electron oxidation of hydroxylamine.

Electron Donation in O_2-Evolving PSII Membranes in the Presence of Hydroxylamine. The results of Figure 2b suggest that formation of nitroxide radicals during illumination does not occur in hydroxylamine-treated, O_2-evolving preparations. This result is consistent with the finding of Bennoun and Joliot (1969) that hydroxylamine at low concentrations does not appreciably affect the rate of O_2 evolution under conditions of continuous turnover. Thus, we conclude that hydroxylamine interacts with functional O_2-evolving centers in a different manner from that observed in Tris-washed PSII membranes.

The dark reaction of hydroxylamine (200 μM) with the O_2-evolving center in the S_1 state at 0 °C requires about 5 min to proceed to completion under our conditions (Beck & Brudvig, 1987a). As shown in Figure 4, the EPR spectrum obtained from dark-adapted, untreated PSII membranes (Figure 4a) is essentially the same as that observed in dark PSII membranes after completion of the reaction with 200 μM hydroxylamine (Figure 4b). In particular, the lack of an EPR signal from Mn(II) in Figure 4b indicates that the hydroxylamine treatment does not cause release of functional Mn from the O_2-evolving center. However, longer incubation times with higher concentrations of hydroxylamine than used here displace Mn (Cheniae & Martin, 1971), and the displaced Mn exhibits a six-line Mn(II) EPR signal (data not shown).

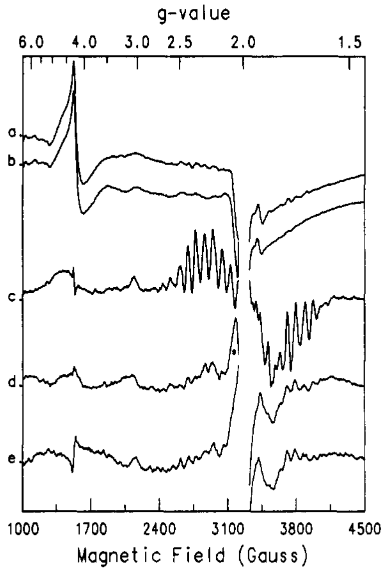


FIGURE 4: Effect of hydroxylamine treatment on low-temperature EPR signals exhibited by PSII membranes at pH 6.0. The $g = 2.0$ region of each spectrum is not shown owing to interference from EPR signal II_s. PSII membranes (4 mg of chlorophyll/mL) were treated with 100 μM DCMU. Spectra obtained with dark-adapted samples, prior to illumination: (a) untreated, control PSII membranes; (b) PSII membranes treated with 200 μM hydroxylamine, incubated for 5 min at 0 °C prior to freezing in liquid N_2. Difference spectra, obtained by subtraction of the spectra obtained with dark-adapted samples from the spectra obtained with the same samples following illumination at 200 K for 120 s: (c) untreated PSII membranes; (d) PSII membranes treated in darkness with 200 μM hydroxylamine for 5 min at 0 °C prior to freezing in liquid N_2; (e) PSII membranes treated with 200 μM hydroxylamine, as in (d), except that 250 μM DCBQ was added after the hydroxylamine treatment, and the sample was incubated an additional 4 min in darkness at 0 °C prior to freezing in liquid N_2. EPR spectrometer conditions: microwave frequency, 9.0 GHz; microwave power, 200 μW; field modulation amplitude, 20 G; sample temperature, 8 K. Each spectrum is the average of two scans.

After illumination at 200 K for 120 s, untreated PSII membranes exhibit a large S_2-state multiline EPR signal centered at $g = 2.0$ (Figure 4c). Superimposed on the hyperfine lines of the multiline EPR signal is an EPR signal at $g = 1.9$, arising from the reduced primary quinone electron acceptor of PSII, Q_A^-, which is magnetically coupled to an Fe(II) ion (Rutherford & Zimmermann, 1984). In addition, a small amount of an EPR signal at $g = 3.0$, arising from high-potential ferricytochrome b_{559}, is produced by the illumination. In untreated PSII membranes, it is apparent that the predominant result of a single charge separation produced by illumination at 200 K is a reduction of Q_A and an oxidation of the Mn complex, producing the multiline and $g = 1.9$ EPR signals that are present in the difference spectrum.

In hydroxylamine-treated PSII membranes, however, illumination at 200 K produces a different result (Figure 4d). Although an $Fe(II)Q_A^-$ EPR signal is observed in Figure 1d, showing that a stable charge separation occurs as a result of the illumination, only a small amount of the S_2-state multiline EPR signal is formed. This finding indicates that hydroxylamine reacts under our conditions with nearly all of the O_2-evolving centers in the PSII membrane sample during a 5-min incubation in darkness and inhibits the formation of the S_2-state multiline EPR signal.

An additional radical EPR signal, ranging from approximately 3100 to 3400 G, is observed in hydroxylamine-treated PSII membranes after illumination at 200 K (asterisk in Figure 4d) and is mostly obscured by EPR signal II_s. The extremely small size of this radical EPR signal, which is not observable when EPR signal II$_s$ is recorded on scale under nonsaturating conditions, shows that it arises in a negligible fraction of the reaction centers. The origin of this radical EPR signal is not known at present, although it is also observed in hydroxylamine-treated, Tris-washed PSII membranes and, therefore, may be generated by the electron-acceptor side of PSII during illumination at low temperature.

The reduced yield of the S_2-state multiline EPR signal in hydroxylamine-treated PSII membranes suggests either that the O_2-evolving center is reduced by hydroxylamine in the dark to a lower oxidation state or that a bound hydroxylamine molecule is oxidized during the illumination, thereby interfering with the normal course of electron donation from the Mn complex to P680$^+$. The latter explanation is involved in the mechanisms for the interaction of hydroxylamine with the O_2-evolving center that were discussed in the introduction. Each of these mechanisms involves the binding of hydroxylamine to a site in the O_2-evolving center. Moreover, at least in the model of Förster and Junge (1985c, 1986), which has been supported by Hanssum and Renger (1985), a cooperative and reversible binding of hydroxylamine to the O_2-evolving center has been proposed.

In order to test the reversibility of the reaction of hydroxylamine with the O_2-evolving center in the S_1 state, we performed experiments in which the hydroxylamine left unreacted at the end of the dark incubation period at 0 °C is consumed by an excess of DCBQ. The reaction of DCBQ with hydroxylamine proceeds to completion under our conditions within 4 min, as judged from spectrophotometric detection at 420 nm (data not shown) of the product 2,5-dichloro-*p*-benzoquinone oxime (Coombes, 1979). PSII membranes that were treated with 200 μM hydroxylamine and incubated for 5 min in the dark were then treated with 250 μM DCBQ for 4 min, in order to consume the excess hydroxylamine. Under these conditions illumination at 200 K does not yield a larger S_2-state multiline EPR signal than is observed in the absence of DCBQ (compare parts d and e of Figure 4). The benzoquinone oxime itself is not inhibitory to the O_2-evolving center, as judged from the ability to generate a normal yield of the S_2-state multiline EPR signal when 200 μM hydroxylamine is added to PSII membranes *after* the addition of 250 μM DCBQ (data not shown).

Under our conditions, then, the state produced in the dark by reaction of the S_1 state with hydroxylamine cannot be reversed to the normal S_1 state by simply removing the hydroxylamine. This finding contradicts the conclusions of Förster and Junge (1985c); however, Kok and Velthuys (1977) found that the two-flash delay in the O_2-evolution pattern in hydrazine-treated thylakoid membranes was not extensively reversed by a 2-h incubation in the presence of ferricyanide or benzoquinone, in agreement with our findings. Moreover, Bouges (1971) found that the effects of hydroxylamine on the flash-induced O_2-evolution pattern in *Chlorella* and in spinach thylakoid membranes could not be reversed by removing the hydroxylamine in darkness.

Owing to the lack of reversibility under our conditions, then, the reaction of hydroxylamine in the S_1 state does not seem to involve an equilibrium binding to a site in the O_2-evolving center. We consider now, as an alternative, whether the reaction of hydroxylamine involves reduction of a site on the electron-donor side of PSII. One possibility involves the reduction by hydroxylamine of a site other than the Mn complex. This site, in turn, might reduce the Mn complex to the S_0 state after formation of the S_2 state during illumination. We can eliminate cytochrome b_{559} from consideration since it is predominantly in the ferrous state in dark PSII membrane samples under our conditions. However, the tyrosine cation radical species D$^+$, from which EPR signal II$_s$ arises (Berry & Babcock, 1987), has been shown to oxidize the S_0 state to the S_1 state in the dark (Styring & Rutherford, 1987). It is possible, then, that the D$^+$ species could be involved in redox-state changes that occur in hydroxylamine-treated PSII membranes. However, the results shown in Figure 5 indicate that EPR signal II$_s$ is not reduced in intensity after the reaction of hydroxylamine with the O_2-evolving center has gone to completion. This finding rules out the involvement of the D$^+$ species in the hydroxylamine reaction.

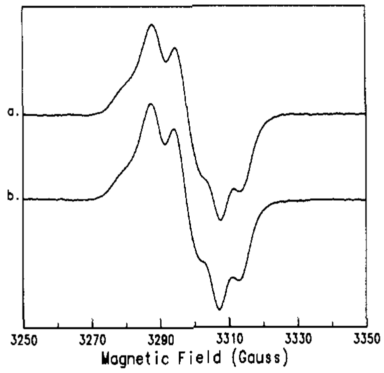


FIGURE 5: Effect of hydroxylamine on EPR signal II$_s$ in PSII membranes (4 mg of chlorophyll/mL) at pH 6.0. EPR spectrometer conditions were as described for Figure 4 except that the microwave power was 50 μW and the sample temperature was 60 K. (a) Untreated, control PSII membranes; (b) PSII membranes treated with 200 μM hydroxylamine and incubated at 0 °C for 5 min prior to freezing in liquid N_2.

We are, therefore, led to assess the involvement of other possible electron donors to P680$^+$ in hydroxylamine-treated PSII membranes. This was accomplished by measuring the yield of the chlorophyll cation radical, high-potential ferricytochrome b_{559}, S_2-state $g = 4.1$ and S_2-state multiline EPR signals as a function of the illumination temperature. If hydroxylamine behaves as an electron donor to P680$^+$, in lieu of the endogenous electron donors, then the yields of these EPR signals will be reduced in the presence of hydroxylamine. Experiments were conducted employing illumination temperatures ranging from 77 to 273 K; DCMU was present in order to restrict PSII reaction centers to a single charge separation. The EPR signals were measured relative to the yield of the Fe(II)Q_A^- EPR signal at $g = 1.9$, which provides a relative measure of the number of stable charge separations (de Paula et al., 1985).

Over the entire illumination temperature range from 77 to 273 K, the S_2-state multiline and $g = 4.1$ EPR signals are not produced in hydroxylamine-treated PSII membrane samples nor are any nitroxide radical EPR signals formed. Figure 6 shows the result of illumination of untreated and of hydroxylamine-treated PSII membranes at 130 K, as an example of the data obtained. The yields of stable charge separations are equivalent in both samples, as judged from the similar intensities of the $g = 1.9$ EPR signal from Fe(II)Q_A^-. As previously observed (Casey & Sauer, 1984; de Paula et al., 1985), illumination at 130 K of untreated PSII membranes produces a large yield of the S_2-state $g = 4.1$ EPR signal, an EPR signal at $g = 3.0$ from high-potential ferricytochrome b_{559}, and very little S_2-state multiline EPR signal (Figure 6a). In contrast, after treatment with 200 μM hydroxylamine, PSII membranes do not exhibit either the S_2-state $g = 4.1$ or the S_2-state multiline EPR signal (Figure 6b). Casey and Sauer

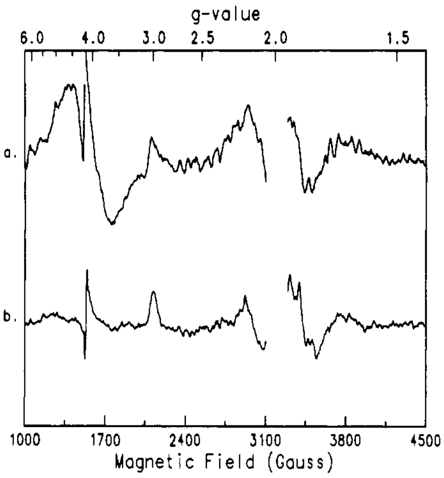


FIGURE 6: Effect of hydroxylamine on the yield of the S_2-state g = 4.1 EPR signal produced by illumination at 130 K of PSII membranes at pH 6.0. PSII membranes (4 mg of chlorophyll/mL) were treated with 100 μM DCMU; the samples were illuminated at 130 K for 10 min. EPR spectrometer conditions were as described for Figure 4. (a) Untreated PSII membranes; (b) PSII membranes treated with 200 μM hydroxylamine at 0 °C for 5 min prior to freezing in liquid N_2. The spectra shown are the difference between the spectrum obtained following illumination and the spectrum obtained prior to illumination.

(1984) obtained a similar result in hydroxylamine-treated PSII membranes.

In hydroxylamine-treated PSII membranes, the only EPR signals that are produced in significant yield by illumination over the 77–273 K range are from high-potential ferricytochrome b_{559} and the chlorophyll cation radical. The chlorophyll cation radical signal can be attributed to the small fraction of O_2-evolving centers that has lost the 17- and 23-kDa extrinsic polypeptides during isolation and handling; the low-potential ferricytochrome b_{559} EPR signal is exhibited by these sites in dark spectra (de Paula et al., 1986b), as can be seen by the small amount of an EPR signal having a g = 3.0 turning point in Figure 4b. Small amounts of the chlorophyll cation radical EPR signal are also observed following low-temperature illumination in untreated PSII membrane samples, in yields commensurate with the amount of the EPR signal from low-potential ferricytochrome b_{559} observed in dark spectra.

In PSII centers that retain the extrinsic polypeptides, however, high-potential ferrocytochrome b_{559} is oxidized at low temperatures during illumination, as was first shown by Knaff and Arnon (1969). Figure 7 compares the relative yield of high-potential ferricytochrome b_{559} in untreated and in hydroxylamine-treated PSII membranes, obtained over the 77–273 K illumination temperature range. As the illumination temperature increases, the yield of high-potential ferricytochrome b_{559} decreases monotonically in both types of samples; note, however, that in the hydroxylamine-treated samples, the yield levels out at about 30% relative intensity at illumination temperatures higher than about 200 K. In untreated PSII membrane samples, the decrease in yield of ferricytochrome b_{559} is matched by a proportionate increase in the yield of the S_2-state multiline EPR signal. These two species together account for the oxidizing equivalent generated by the charge separation (de Paula et al., 1985). However, no EPR signals are formed in response to the decrease in yield of ferricytochrome b_{559} in hydroxylamine-treated samples.

The measurements in Figure 7 are made relative to the yield of the g = 1.9 Fe(II)Q_A^- EPR signal, which accounts for the reducing equivalents produced during the illumination. The decrease in the yield of ferricytochrome b_{559} as the illumination temperature is raised in hydroxylamine-treated PSII membranes cannot, therefore, be attributed to a decrease in the stability of charge separations at the PSII reaction center. Furthermore, an increasing yield of the chlorophyll cation radical or nitroxide radical EPR signals is not observed as the illumination temperature is raised. By process of elimination, the undetected electron donor must either be the Mn complex or a bound hydroxylamine molecule that undergoes a two-electron oxidation, so as to avoid the formation of a nitroxide radical.

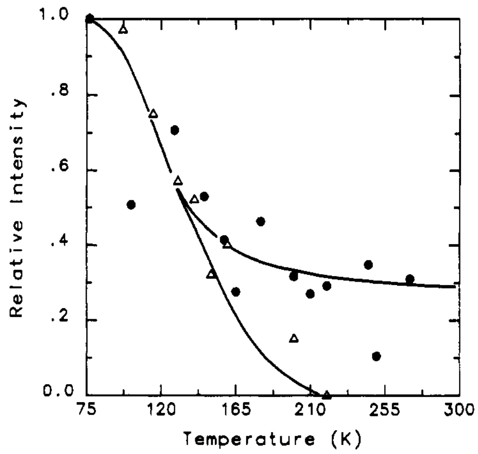


FIGURE 7: Yield of ferricytochrome b_{559}, as measured from the intensity of the g = 3.0 turning point, after illumination of untreated PSII membranes (△) and of PSII membranes treated with 200 μM hydroxylamine (●) at pH 6.0. Hydroxylamine-treated PSII membrane samples were incubated for 5 min at 0 °C, in darkness, prior to freezing. The data from untreated PSII membranes are those obtained by de Paula et al. (1985). The yield of the Fe(II)Q_A^- EPR signal at g = 1.9 following illumination was normalized in each spectrum so as to reflect an equivalent yield of stable charge separations at the PSII reaction center. The yield of ferricytochrome b_{559} was obtained from the height of the g = 3.0 turning point from peak to base line. EPR spectrometer conditions were as described for Figure 4.

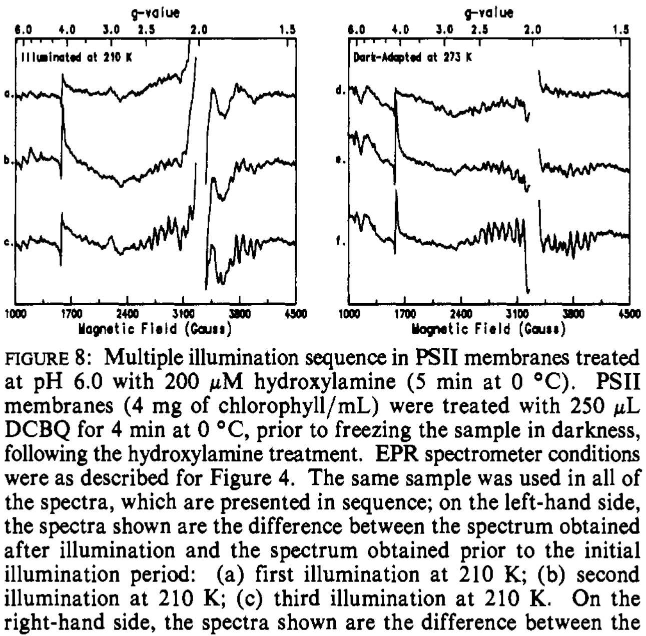


FIGURE 8: Multiple illumination sequence in PSII membranes treated at pH 6.0 with 200 μM hydroxylamine (5 min at 0 °C). PSII membranes (4 mg of chlorophyll/mL) were treated with 250 μL DCBQ for 4 min at 0 °C, prior to freezing the sample in darkness, following the hydroxylamine treatment. EPR spectrometer conditions were as described for Figure 4. The same sample was used in all of the spectra, which are presented in sequence; on the left-hand side, the spectra shown are the difference between the spectrum obtained after illumination and the spectrum obtained prior to the initial illumination period: (a) first illumination at 210 K; (b) second illumination at 210 K; (c) third illumination at 210 K. On the right-hand side, the spectra shown are the difference between the spectrum obtained following illumination and the subsequent dark incubation at 0 °C and the spectrum obtained prior to the initial illumination period: (d) after the first illumination; (e) after the second illumination; (f) after the third illumination. Note that the vertical scaling factor used in this figure differs from that used in Figure 4; the yield of the S_2-state multiline signal in (c) is about 75% of that observed after a single illumination at 210 K in an untreated, control sample (as in Figure 4c).

The results suggest that the Mn complex is left in an EPR-silent oxidation state following illumination in hydroxylamine-treated PSII membranes. In order to determine which S state is present prior to illumination, we performed an experiment involving sequential illuminations at 210 K (Figure 8). The technique employed involves the reoxidation of Q_A^- by excess DCBQ after illumination. It was observed previously that the $Fe(II)Q_A^-$ EPR signal decays quantitatively in PSII membranes illuminated at 210 K if the sample is warmed in darkness for 1 min to about 0 °C in the presence of excess DCBQ (de Paula et al., 1986a). This reoxidation of Q_A^- allows another charge separation to proceed at low temperature. In order to use this technique in hydroxylamine-treated PSII membranes, we prepared samples in which excess DCBQ was added after the reaction with hydroxylamine had gone to completion. As discussed earlier, this treatment also consumes the unreacted hydroxylamine present in the sample.

Figure 8 shows that the S_2-state multiline EPR signal is observed in significant yield in hydroxylamine-treated PSII membranes only after the third illumination at 210 K. The yield of the S_2-state multiline EPR signal in Figure 8c, after the third illumination, is about 75% of that obtained in an untreated, control sample (as in Figure 4c). Considering that a small fraction of ferrocytochrome b_{559} is oxidized in each charge separation and considering that each dark incubation at 0 °C allows 5–10% of the reaction centers to back-react (de Paula et al., 1986a; Beck & Brudvig, 1986), the yield of the S_2-state multiline EPR signal obtained in Figure 8e is the maximum that can be expected. Thus, within experimental error, all of the O_2-evolving centers in the sample used in Figure 8 are delayed by 2 quanta as a result of the treatment with hydroxylamine in the dark. The small yield of the S_2-state multiline EPR signal present following the second illumination (Figure 8b) is due to the absence of DCMU in these samples, which allows a small number of centers to undergo two charge separations at 210 K. It is also apparent from the spectra in Figure 8 that the Mn complex does not exhibit any new EPR signals in the formal S_{-1}, S_0, and S_1 states.

DISCUSSION

The interaction of hydroxylamine at low concentrations with the electron-donor side of PSII evidently causes an interruption in the accumulation of oxidizing equivalents required for the oxidation of H_2O to O_2. In the past, hydroxylamine has been thought to behave as an alternate substrate for the O_2-evolving center, competing with H_2O for oxidizing equivalents and forming N_2 as product (Radmer & Ollinger, 1983). Recent results, however, make it seem unlikely that hydroxylamine functions as a substrate analogue since it appears to reduce the Mn complex in the dark after binding at the Cl^--binding site of the O_2-evolving center (Beck & Brudvig, 1987a). Moreover, the reversibility of the interaction of hydroxylamine with the O_2-evolving center appears to depend on the concentration of hydroxylamine added to the PSII membrane sample. Several investigations, in which low concentrations of hydroxylamine were used, have indicated that the effects of hydroxylamine on the proton-release and O_2-evolution flash patterns can be reversed by washing out the hydroxylamine (Förster & Junge, 1985c; Hanssum & Renger, 1985). These observations have led to the proposal that hydroxylamine binds reversibly to the S_1 state and that the oxidation of hydroxylamine is triggered by the formation of the S_2 state, as in eq 6. In contrast, we find that hydroxylamine reacts irreversibly with the O_2-evolving center in the dark without releasing Mn(II) ions and that this reaction suppresses the formation of the S_2-state $g = 4.1$ and multiline EPR signals on the first low-temperature illumination. In order to account for these results, the reaction of hydroxylamine with the O_2-evolving center must proceed in two steps: first, hydroxylamine must bind reversibly to a site in the O_2-evolving center and, second, an irreversible reaction of hydroxylamine occurs. We identify the irreversible reaction as a two-electron reduction of the Mn complex by hydroxylamine. Depending on the concentration of hydroxylamine added to the PSII membrane sample, the irreversible reaction may proceed to a greater or lesser extent.

In another paper (Beck & Brudvig, 1987a), we found that the rate of hydroxylamine's reaction with the O_2-evolving center in the S_1 state depends inversely on the Cl^- concentration. To account for these results, we suggested that hydroxylamine first binds reversibly at the Cl^--binding site of the O_2-evolving center and then reacts irreversibly with the Mn complex, as outlined in eq 7 and 8. The equilibrium

$$\mathrm{NRR'OH} + \mathrm{S_1(Cl^-)} \overset{K_{eq}}{\rightleftharpoons} \mathrm{S_1(NRR'OH)} + \mathrm{Cl^-} \quad (7)$$

$$\mathrm{S_1(NRR'OH)} \rightarrow \mathrm{S_{-1}} + \text{products} \quad (8)$$

constant K_{eq} governs the displacement of Cl^- by the hydroxylamine species NRR'OH from the O_2-evolving center; once bound, the hydroxylamine species was proposed to react with the Mn complex, forming the S_{-1} state, thereby suppressing the formation of the S_2-state multiline EPR signal on the first charge separation.

The nature of the S_{-1} state shown in eq 8 was not made clear by our previous experiments. The loss of two oxidizing equivalents by the O_2-evolving center after incubation in the dark with hydroxylamine could involve several mechanisms, three of which were discussed in the introduction. In these three models, the final dark state of the O_2-evolving center involves an equilibrium binding of hydroxylamine to a site in the O_2-evolving center. Through the results of experiments discussed in this paper, however, we showed that the normally light-inducible S_2-state multiline EPR signal remains suppressed following hydroxylamine treatment even when the excess hydroxylamine is removed chemically (Figure 4). Thus, when the reaction has gone to completion, a stable product must be formed. This result argues strongly against the involvement of exchangeable hydroxylamine molecules in the S_{-1} state.

Several possibilities exist for the stable product formed by the reaction of hydroxylamine with the O_2-evolving center in the S_1 state: (1) a covalent adduct of hydroxylamine, such as an oxime, with a protein functional group; (2) a covalent adduct of one or more hydroxylamine molecules with the Mn complex; (3) a reduced component of the electron-donor side of PSII other than the Mn complex; (4) a reduced Mn complex. We will consider these possibilities in turn.

A covalent adduct of hydroxylamine with a protein functional group in the O_2-evolving center, such as an oxime, would be expected to be relatively stable under the weakly acidic conditions present in our experiments. However, oximes are unlikely to be readily oxidizable by PSII. For instance, in Tris-washed PSII membranes, in which many exogenous electron donors can be readily photooxidized (Yerkes & Babcock, 1980), the 2,5-dichloro-*p*-benzoquinone oxime formed by reaction of DCBQ and hydroxylamine proved unable to serve as an electron donor, as judged by DCIP photoreduction assays (data not shown). Additionally, we detected no difference in the behavior of PSII membrane samples whether treated with hydroxylamine, *N*-methylhydroxylamine, or *N*,*N*-dimethylhydroxylamine, except for the kinetics of the reaction (Beck & Brudvig, 1987a). One would expect different chemistries of protein adduct formation in these three cases.

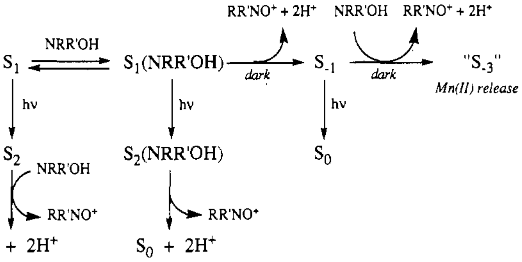
Thus, it seems unlikely that a hydroxylamine adduct with a protein functional group in the O_2-evolving center can be the source of reducing equivalents that cause the delay by 2 quanta in the advance of the S states.

The covalent coordination of one or more hydroxylamine molecules to the Mn complex could account for the experimental evidence we obtained. For instance, a single hydroxylamine molecule tightly bound to the Mn complex in the S_1 state might be oxidized by two electrons upon formation of the S_2 state, leaving the O_2-evolving center in the S_0 state after the reaction. A nitroso compound would be left as the nitrogen-containing product, which would be consistent with our failure to observe nitroxide radicals in illuminated, hydroxylamine-treated PSII membranes. Alternatively, two hydroxylamine molecules tightly bound to the Mn complex might each be oxidized by one electron and combine within the active-site region of the protein, producing N_2 as the final product. This scenario is similar to that suggested by Radmer and Ollinger (1982, 1983) and would also avoid production of EPR-detectable nitroxide radicals.

However, several points argue against the formation of a stable hydroxylamine adduct with the Mn complex in the S_1 state. The oxidation of the covalently bound hydroxylamine molecules would have to be triggered by the formation of the S_2 state during illumination in order to account for the delay in the advance of the S states. We performed an extensive series of experiments over a wide illumination temperature range and failed to see formation of either the S_2-state $g = 4.1$ or multiline EPR signals or of signals that could be attributed to a modified Mn complex in the S_2 state. In order to prevent our observation of a trapped population of the S_2-state intermediate, the bound hydroxylamine molecules would have to react extremely rapidly, even at 130 K. Nevertheless, this may not be unreasonable given the known rapidity of electron-transfer reactions in photosynthetic reaction centers even at very low temperatures. What is most difficult to explain is the required stability of the bound hydroxylamine molecules when coordinated to the Mn complex in the S_1 state. First, such a bound hydroxylamine cannot dissociate significantly following removal of the free hydroxylamine from the sample. This implies a binding constant that is inconsistent with studies done at low hydroxylamine concentrations which show reversible binding. Second, the Mn tetramer complex is likely to be in either a 3Mn(III)–Mn(IV) or a 3Mn(IV)–Mn(III) configuration in the S_2 state (de Paula et al., 1987). In the S_1 state, then, the Mn complex is either a 4Mn(III) or a 2Mn(III)–2Mn(IV) system. It seems unlikely that hydroxylamine molecules can remain stable when bound to such a high-oxidation-state complex, especially considering that hydroxylamine is a very good reductant.

We turn next to the possibility that hydroxylamine reduces a site on the electron-donor side of PSII in the dark. The present results show that D^+, the only known reducible site on the dark-adapted electron-donor side other than the Mn complex itself, is not reduced by hydroxylamine treatment as judged from the intensity of EPR signal II_s. This raises the possibility that a heretofore undetected, EPR-silent, and redox-active site exists on the electron-donor side of PSII that reacts with hydroxylamine in darkness. Upon generation of the S_2 state during illumination, this site would have to be oxidized by two electrons by the Mn complex in order to account for O_2-evolution and proton-release results. This possibility is consistent with the observation by Guiles et al. (1987) that the Mn X-ray absorption edge energy is not affected by treatment with hydroxylamine in the dark and that a single charge separation results in a shift of the edge to lower energy. Again, however, this possibility involves an S_2-state intermediate that was not detected in our low-temperature EPR experiments. Further, it is difficult to understand why the Mn complex would not be reduced prior to illumination by this hypothetical, hydroxylamine-reduced, redox-active site if one considers the requirement that this site be located close enough to the Mn complex that a rapid electron-transfer reaction could occur between them upon formation of the S_2 state.

Scheme I: Model for the Reaction of Hydroxylamine and *N*-Alkyl-Substituted Analogues with the Electron-Donor Side of PSII in O_2-Evolving PSII Membranes

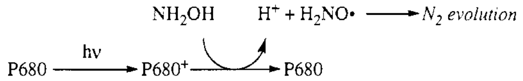


We conclude instead that the dark reaction of hydroxylamine with the O_2-evolving center involves a two-electron reduction of the Mn complex. A possible mechanism, consistent with the available results, is shown in Scheme I. We propose that the irreversible reaction of hydroxylamine involves a reduction of the Mn complex in the dark by two electrons from the S_1 state to the S_{-1} state. The S_{-1}-state intermediate is proposed to be a discrete oxidation state of the Mn complex, rather than the S_0 or S_1 state with bound hydroxylamine molecule(s); a similar proposal was made previously by Saygin and Witt (1985). As we have proposed (Beck & Brudvig, 1987a), an additional molecule of hydroxylamine could react in the same manner with the Mn complex in the S_{-1} state, causing a further reduction to an unstable S_{-3} state, which would contain Mn(II) ions that are expected to be labile. Thus, the proposed mechanism argues that the two-flash delay in the advance of the S states in the presence of low concentrations of hydroxylamine and the displacement of functional Mn ions that occurs at high concentrations of hydroxylamine are caused by identical mechanisms involving two-electron reductions of the Mn complex by hydroxylamine.

The results of Andréasson and Hansson (1987) strongly suggest that another mode of hydroxylamine's interaction with the electron-donor side of PSII involves a reaction with the S_2 state. The final effects of the reaction of hydroxylamine with either the S_1 or the S_2 state appear to be indistinguishable; in both cases, the S_0 state is the product of a single charge separation. Thus, in Scheme I, we show that a two-electron reduction of the Mn complex after formation of the S_2 state may also yield the S_0 state.

As shown in Scheme I, a two-electron reduction of the Mn complex to the S_0 state may also result upon photooxidation of the S_1(NRR′OH) intermediate. This pathway may be especially important when the dark reduction of the Mn complex has not gone to completion. From our previous results (Beck & Brudvig, 1987a), the rate constants for formation of the S_{-1} state in the presence of hydroxylamine are rather small and depend inversely on the Cl^- concentration. Thus, under the conditions of Förster and Junge (1985c), for instance, the rate of the irreversible two-electron reduction of the Mn complex from the S_1 state to the S_{-1} state would have been very slow owing to the low concentrations of hydroxylamine and reaction centers employed. This could account for

Scheme II: Model for the Reaction of Hydroxylamine with the Electron-Donor Side of PSII in Tris-Washed PSII Membranes



the observed reversibility of the effects of hydroxylamine on the flash-induced proton yield patterns. Further, in the Mn X-ray absorption edge experiment of Guiles et al. (1987), the very high concentration of PSII membranes employed would have lessened the extent of the dark reduction of the Mn complex to the S_{-1} state under the low hydroxylamine concentration and relatively short dark incubation conditions employed. This explanation is consistent with the observed lack of change in the position of the Mn X-ray absorption edge upon dark hydroxylamine treatment and with the substantial yield of the S_2-multiline EPR signal after an illumination at 190 K (Guiles et al., 1987). The net movement of the Mn X-ray absorption edge to lower energy after the illumination at 190 K would reflect the two-electron reduction of the Mn site from the S_2 state to the S_0 state in those O_2-evolving centers that were trapped in the S_1(NRR′OH) intermediate state upon freezing the sample.

Each of the three pathways suggested for the reaction of hydroxylamine with the O_2-evolving center shown in Scheme I involves two-electron oxidation reactions of hydroxylamine. It is likely that analogous two-electron reactions can account for the similar behavior of the O_2-evolving center in the presence of hydrazine (Förster & Junge, 1986). In Tris-washed PSII membranes, however, two-electron redox reactions are not possible owing to the loss of the Mn complex. Instead, Tris-washed PSII membranes perform light-driven, one-electron oxidation reactions employing hydroxylamine molecules as substrate. We show in Scheme II that the product of the one-electron oxidation of hydroxylamine by Tris-washed PSII membranes is a nitroxide radical, which we detected in the case of the reaction of *N*,*N*-dimethylhydroxylamine. As was pointed out by Radmer (1979), hydroxylamine radicals can recombine to form N_2 [for a review on this chemistry, see Eisner and Kirowa-Eisner (1979)]. Because we failed to observe formation of the dimethyl nitroxide radical in O_2-evolving PSII membranes treated with *N*,*N*-dimethylhydroxylamine, it seems likely that the N_2 detected by Radmer (1979) in hydroxylamine-treated thylakoid membranes arose from one-electron oxidation reactions in damaged centers, which may have become depleted of functional Mn during the incubation period in the dark with hydroxylamine. Saygin and Witt (1985) have also questioned whether the N_2 evolved in hydroxylamine-treated thylakoid membranes might originate from inactive centers; they point out that if hydroxylamine reduces the S_1 state to the S_{-1} state in the dark, N_2 cannot be evolved on the first flash in intact O_2-evolving centers.

There is substantial experimental evidence for the one-electron oxidation mechanism shown in Scheme II in the case of hydrogen peroxide: Velthuys and Kok (1978b) noted a photochemical dismutation of hydrogen peroxide that occurred only in Tris-washed thylakoid membranes. This dismutase activity produced O_2 on every flash of light, in strict analogy to the N_2 yielded on each flash in hydroxylamine-treated thylakoid membranes (Radmer, 1979) and in hydrazine-treated, Tris-washed thylakoid membranes (Radmer & Ollinger, 1981). Hence, we propose that the mechanism outlined in Scheme II is operative only in Mn-depleted PSII centers.

Registry No. NH_2OH, 7803-49-8; Mg, 7439-95-4; H_2O, 7732-18-5; O_2, 7782-44-7.

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Anomalous Uncoupling of Photophosphorylation by Palmitic Acid and by Gramicidin D[†]

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ABSTRACT: Palmitic acid and gramicidin D at low concentrations uncouple photophosphorylation in a mechanism that is inconsistent with classical uncoupling in the following properties: (1) ΔpH, H$^+$ uptake, or the transmembrane electric potential is not inhibited. (2) O$_2$ evolution is stimulated under nonphosphorylating conditions but slightly inhibited in the presence of adenosine 5′-diphosphate + inorganic phosphate (P$_i$). (3) Light-triggered adenosine 5′-triphosphate (ATP)–P$_i$ exchange is hardly affected, and ATPase activity is only slightly stimulated. (4) ATP-induced ΔpH formation is selectively inhibited. This characteristic uncoupling is observed only when the native coupling sites of the electron transport system are used for energization such as for methylviologen-coupled phosphorylation. With pyocyanine, which creates an artificial coupling site, 1000-fold higher gramicidin D and higher palmitic acid concentrations are required for inhibition, and the inhibition is accompanied by a decrease in ΔpH. Moreover, comparison between photosystem 1 and photosystem 2 electron transport and the effects of membrane unstacking suggest that low gramicidin D preferentially inhibits photosystem 2, while palmitic acid inhibits more effectively photosystem 1 coupling sites. The inhibitory capacity of fatty acids significantly drops when the chain length is reduced below 16 hydrocarbons or upon introduction of a single double bond in the hydrocarbon chain. It is suggested that palmitic acid and gramicidin D interfere with a direct H$^+$ transfer between specific electron transport and the ATP synthase complexes, which provides an alternative coupling mechanism in parallel with bulk to bulk $\Delta\tilde{\mu}_{H^+}$. The sites of inhibition seem to be located in chloroplast ATP synthase, photosystem 2, and the cytochrome b_6f complexes.

Several mechanisms have been proposed to explain energy transduction leading to adenosine 5′-triphosphate (ATP) formation in photosynthetic and respiratory systems. According to the chemiosmotic hypothesis (Mitchell, 1966, 1979), which has been widely accepted, ATP formation is mediated by a transmembrane proton electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$) that is generated by electron (e$^-$) transport reactions. An alternative hypothesis, proposed by Williams (Williams 1959, 1961, 1976), suggested that protons are released within the

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