Binding of Amines to the O_2-Evolving Center of Photosystem II†

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ABSTRACT: The binding of several primary amines to the O_2-evolving center (OEC) of photosystem II (PSII) has been studied by using low-temperature electron paramagnetic resonance (EPR) spectroscopy of the S_2 state. Spinach PSII membranes treated with NH_4Cl at pH 7.5 produce a novel S_2-state multiline EPR spectrum with a 67.5-G hyperfine line spacing when the S_2 state is produced by illumination at 0 °C [Beck, W. F., de Paula, J. C., & Brudvig, G. W. (1986) *J. Am. Chem. Soc. 108*, 4018–4022]. The altered hyperfine line spacing and temperature dependence of the S_2-state multiline EPR signal observed in the presence of NH_4Cl are direct spectroscopic evidence for coordination of one or more NH_3 molecules to the Mn site in the OEC. In contrast, the hyperfine line pattern and temperature dependence of the S_2-state multiline EPR spectrum in the presence of tris(hydroxymethyl)aminomethane, 2-amino-2-ethyl-1,3-propanediol, or CH_3NH_2 at pH 7.5 were the same as those observed in untreated PSII membranes. We conclude that amines other than NH_3 do not readily bind to the Mn site in the S_2 state because of steric factors. Further, NH_3 binds to an additional site on the OEC, not necessarily located on Mn, and alters the stability of the S_2-state $g = 4.1$ EPR signal species. The effects on the intensities of the $g = 4.1$ and multiline EPR signals as the NH_3 concentration was varied indicate that both EPR signals arise from the same paramagnetic site and that binding of NH_3 to the OEC affects an equilibrium between two configurations exhibiting the different EPR signals. The results of this paper support the proposal that a single Mn site functions on the electron donor side of PSII in the mechanism of photosynthetic O_2 evolution; the Mn site functions both in the storage of oxidizing equivalents and, considering the steric selectivity of the Mn site for the coordination of small Lewis bases, in binding and oxidation of substrate H_2O molecules.

The oxidation of H_2O by photosystem II (PSII)[1] is performed by an O_2-evolving center (OEC) that contains four Mn ions (Amesz, 1983; Murata et al., 1984). Sequential absorption of photons by the PSII reaction center advances the OEC through its five oxidation states S_i, $i = 0$–4, with release of an O_2 molecule occurring with the conversion of the S_4 state to the S_0 state. The intermediate oxidation states S_2 and S_3 back-react during dark adaptation to form the dark-stable S_1 state (Joliot & Kok, 1975), while the S_0 state is slowly oxidized in the dark to form the S_1 state (Vermaas et al., 1984).

Recent experimental results have confirmed that one function of the Mn site in PSII is the accumulation of the four oxidizing equivalents required to oxidize two H_2O molecules to O_2. Oxidation of Mn during S-state advancement has been observed by using X-ray absorption edge measurements (Goodin et al., 1984) and through observation of the multiline electron paramagnetic resonance (EPR) spectrum exhibited by the Mn site in the S_2 state of the OEC (Dismukes & Siderer, 1981; Zimmermann & Rutherford, 1984). Both techniques indicate that charge-separation events at the PSII reaction center cause oxidation of the Mn site during turnover of the OEC.

Recent EPR experiments have also suggested that the Mn site binds ligands during the S-state cycle. Hansson et al. (1986) observed that $H_2{}^{17}O$-treated PSII membranes produce an S_2-state multiline EPR signal with slightly broadened hyperfine lines after illumination at 200 K. This result shows that one or more O atoms originating from H_2O are coordinated to the Mn site in the S_2 state. Further, PSII membranes treated with NH_4Cl at pH 7.5 produced a multiline EPR spectrum with an altered hyperfine line spacing when the S_2 state was generated by illumination at 0 °C, providing direct spectroscopic evidence for the coordination of NH_3 to the Mn site in the S_2 state (Beck et al., 1986). On the basis of the analogy between the structures of NH_3 and H_2O, the binding of NH_3 to the Mn site probably occurs in lieu of the binding of substrate H_2O molecules (Velthuys, 1975; Sandusky & Yocum, 1984; Beck et al., 1986). Thus, the EPR-detectable Mn site on the electron donor side of PSII appears to function both in storage of oxidizing equivalents and in binding and oxidation of substrate H_2O molecules.

The finding that the structure of the Mn site is altered upon the binding of NH_3, as detected by using the S_2-state multiline EPR signal, raises new possibilities for the study of the coordination chemistry of the Mn site. In particular, ligand-exchange reactions at the Mn site may be detectable by monitoring the S_2-state multiline EPR signal in the presence of inhibitors of photosynthetic O_2 evolution. Previously, the proposals identifying the Mn site as the ligand-binding site of the OEC were based indirectly on the inhibition of O_2 evolution activity in the presence of primary amines and hydroxylamines, which might be expected to coordinate to the Mn site as Lewis bases (Ghanotakis et al., 1983). Sandusky and Yocum (1983, 1984, 1986) found that primary amines inhibit O_2 evolution activity by competing with Cl^- for a site on the OEC, but that NH_3 inhibits activity by binding to still another site independent of the Cl^- concentration. Since the

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[1] Abbreviations: AEPD, 2-amino-2-ethyl-1,3-propanediol; Chl, chlorophyll; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, O_2-evolving center; P680, primary electron donor of PSII; PSII, photosystem II; Q_A, primary quinone electron acceptor in PSII; Tris, tris(hydroxymethyl)aminomethane.

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presence of Cl^- is required for O_2 evolution activity (Kelley & Izawa, 1978), Sandusky and Yocum (1984) proposed that the competition of amines and Cl^- for a binding site on the OEC indicates that the Mn site coordinates one or more Cl^-, perhaps as bridging ligands. Radmer and Ollinger (1983) proposed a model for the shape of the H_2O-binding site of the OEC on the basis of the results of inhibition studies employing various substituted hydroxylamines as H_2O analogues. However, hydroxylamines and primary amines other than NH_3 have not yet been shown to inhibit O_2 evolution activity by binding directly to the Mn site.

In this paper we employ the techniques used in our previous paper on the binding of NH_3 to the Mn site of the OEC (Beck et al., 1986) to monitor ligand exchange reactions at the Mn site in the presence of several primary amines. The steric requirements of the ligand-binding site on Mn are probed by monitoring the S_2-state multiline EPR spectrum in the presence of CH_3NH_2, tris(hydroxymethyl)aminomethane (Tris), and 2-amino-2-ethyl-1,3-propanediol (AEPD). We also consider in more detail the structural changes in the Mn site caused by the binding of NH_3 to the S_1 and S_2 states. The results show that amines other than NH_3 fail to bind to the Mn site in either the S_1 or S_2 states, showing that the probable H_2O-binding site of the OEC on the Mn site is extremely sterically selective for small Lewis bases.

Experimental Procedures

Materials. 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), from Research Organics, and Triton X-100, from Sigma, were used as received. NH_4Cl was obtained from Baker. CH_3NH_2·HCl, 98%, and 2-amino-2-ethyl-1,3-propanediol (AEPD), 97%, were used as received from Aldrich. Tris(hydroxymethyl)aminomethane (Tris) was obtained from Sigma (Trizma base, reagent grade).

Preparation of PSII Membranes. PSII membranes were isolated from market spinach by using a modification of the procedure of Berthold et al. (1981) and were assayed for chlorophyll (Chl) concentration and O_2 evolution activity as described previously (Beck et al., 1985). PSII membranes were stored at 77 K, suspended in a buffer solution containing 20 mM MES–NaOH, 15 mM NaCl, and 30% (v/v) ethylene glycol, pH 6.0. All steps in the isolation procedure were performed in the dark. The PSII membranes used in this study evolved O_2 at 450–800 μmol of O_2 (mg of Chl h)$^{-1}$ at pH 6.0 when illuminated with saturating intensities of light; the activity at pH 7.5 was typically 60–70% of the activity observed at pH 6.0.

EPR Studies. The EPR experiments described in this paper were performed with PSII membrane samples that were not exposed to light before treatment with amines. Previous work has shown that only the resting state of the OEC exists in such extensively dark-adapted samples (Beck et al., 1985); further, only the S_1 state is present in extensively dark-adapted PSII membranes (Vermaas et al., 1984). Experiments were also performed (data not shown) with preilluminated, active-state PSII membrane samples, which exhibit different S_2-state multiline EPR signals and possess altered electron-transfer properties at cryogenic temperatures compared to resting-state samples (Beck et al., 1985), as well as having a mixture of the S_1 and S_0 states in a 3:1 ratio. The results obtained with resting-state PSII membranes were qualitatively the same as those obtained with active-state PSII membranes.

Because it is known that only the free base form of primary amines inhibits photosynthetic O_2 evolution (Ghanotakis et al., 1983), it was necessary to perform all amine-binding experiments with PSII membrane suspensions at pH 7.5, the most alkaline conditions obtainable without irreversible loss of O_2 evolution activity. The pH and Cl^- concentration of PSII membrane suspensions were adjusted through two suspension and recentrifugation cycles in a buffer solution (suspension buffer) containing 20 mM HEPES, pH 7.5, 30% (v/v) ethylene glycol, and either 0.5 or 15 mM NaCl, as noted.

The procedure used for amine-binding experiments was the same as that used before for NH_4Cl treatments (Beck et al., 1986). Briefly, the pellet of PSII membranes obtained from the final centrifugation was resuspended in the suspension buffer to approximately 5 mg of Chl/mL and then NH_4Cl, CH_3NH_2·HCl, Tris, or AEPD was added from a 1.25 M solution in the suspension buffer adjusted to pH 7.5 with H_2SO_4. Either 100 μM DCMU or 250 μM DCBQ was also added from 20 mM solutions in 95% ethanol, where noted. After reagents were mixed with the PSII membranes, the suspensions were incubated in the dark on ice for 10 min in quartz EPR tubes before they were frozen in liquid N_2.

EPR spectra were obtained at 5–25 K with the instrumentation previously described (Beck et al., 1985; de Paula & Brudvig, 1985). Illumination of EPR samples at 130 K, 210 K, or 0 °C was performed as before (de Paula et al., 1985; Beck et al., 1986). A cooled ethanol bath was used for sample temperature control during dark incubations. All EPR spectra shown are difference spectra obtained through computer subtraction of the dark background spectrum from the postillumination or postincubation spectrum obtained under the same measurement conditions.

Results

Binding of Amines to the OEC in the S_2 State. Figure 1 shows the S_2-state multiline EPR spectra that are formed after illumination at 0 °C in untreated and amine-treated PSII membranes. These samples contained DCMU to prevent reoxidation of the primary quinone electron acceptor in PSII, Q_A, by the secondary quinone electron acceptor, Q_B (Joliot & Kok, 1975), so that only the S_2 state could be formed during continuous illumination from the S_1 state initially present. The spectrum from untreated PSII membranes (Figure 1a) is identical with S_2-state EPR spectra observed previously in untreated PSII membranes at higher Cl^- concentrations and in untreated PSII membranes at pH 6.0 (Beck et al., 1986). Superimposed upon the hyperfine line pattern from the S_2-state multiline EPR signal is a broad EPR signal attributed to the reduced form of Q_A, which is magnetically coupled to an Fe(II) ion, the sharpest turning point of which is located at approximately $g = 1.9$ (Rutherford & Zimmerman, 1984).

As was previously shown (Beck et al., 1986), NH_4Cl-treated PSII membranes exhibit an altered S_2-state multiline EPR signal when the S_2 state is produced at 0 °C (Figure 1e). The reduction in the average spacing of the hyperfine lines from 87.5 G, as observed in untreated samples (Figure 1a), to 67.5 G can be attributed to the binding of one or more NH_3 molecules directly to the Mn site of the OEC in the S_2 state.

In contrast, the S_2-state multiline EPR signals obtained in the presence of 100 mM AEPD, 100 mM Tris, or 100 mM CH_3NH_2·HCl, shown in parts b–d of Figure 1, respectively, do not differ significantly from that observed in the untreated sample (Figure 1a). We used only 0.5 mM Cl^- in the ex-

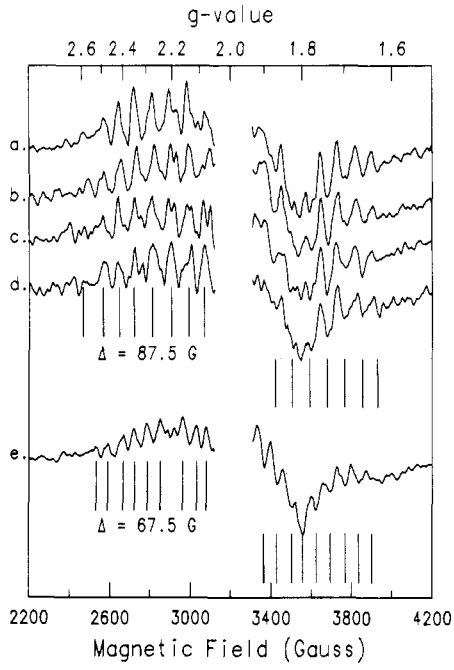


FIGURE 1: Comparison of S_2-state EPR signals produced in PSII membranes at pH 7.5 by illumination at 0 °C for 30 s. The $g = 2.0$ region, which is obscured by interference from EPR signal II_s, is not shown. EPR spectrometer conditions: microwave frequency, 8.9 GHz; microwave power, 200 μW; field modulation frequency, 100 kHz; field modulation amplitude, 20 G; temperature, 8.0 K. PSII membranes (5 mg of Chl/mL) were treated with 100 μM DCMU: (a) untreated PSII membranes (total $[Cl^-] = 0.5$ mM); (b) PSII membranes treated with 100 mM AEPD (total $[Cl^-] = 0.5$ mM); (c) PSII membranes treated with 100 mM Tris (total $[Cl^-] = 0.5$ mM); (d) PSII membranes treated with 100 mM CH_3NH_2·HCl (total $[Cl^-] = 115$ mM); (e) PSII membranes treated with 100 mM NH_4Cl (total $[Cl^-] = 115$ mM). The vertical lines below spectra d and e show the positions of the major hyperfine lines; Δ is the average of the hyperfine line spacings for the indicated spectrum. Each spectrum is the average of two scans.

periments using AEPD or Tris because Sandusky and Yocum (1984) demonstrated that amines inhibit O_2 evolution optimally at low Cl^- concentrations. However, the Cl^- concentration in the CH_3NH_2·HCl-treated sample was 115 mM; lower Cl^- concentrations would necessitate use of the free base form of CH_3NH_2. The relative intensity of the S_2-state multiline EPR signal with respect to the intensity of the EPR spectrum from $Fe^{II}Q_A^-$ is identical in each of the spectra; the intensity of the $Fe^{II}Q_A^-$ EPR signal is a measure of the extent of charge separation in PSII (de Paula et al., 1985), and hence, this observation demonstrates that AEPD, Tris, or CH_3NH_2 does not decrease the yield of the S_2-state multiline EPR signal. The small differences in the hyperfine line pattern in the spectra shown in Figure 1a–d can be attributed to differences in the base line noise pattern. The S_2-state multiline EPR spectra obtained in the presence of AEPD, Tris, or CH_3NH_2 were the same as those shown in Figure 1a–d at higher Cl^- concentrations and, moreover, were independent of the concentration of amine up to 200 mM. At very high concentrations of amine, above about 250 mM, the intensities of the S_2-state multiline and $Fe^{II}Q_A^-$ EPR signals decreased, thereby indicating that a stable charge separation was not obtained.

Experiments were also performed by using illumination at 210 K, rather than at 0 °C, to generate the S_2 state. In all cases, the S_2-state multiline EPR signals were identical with that observed in untreated control samples (data not shown). Further, the addition of AEPD, Tris, or CH_3NH_2 had no effect on the yield of the S_2-state multiline EPR signal when illumination at 210 K was employed. However, as noted below,

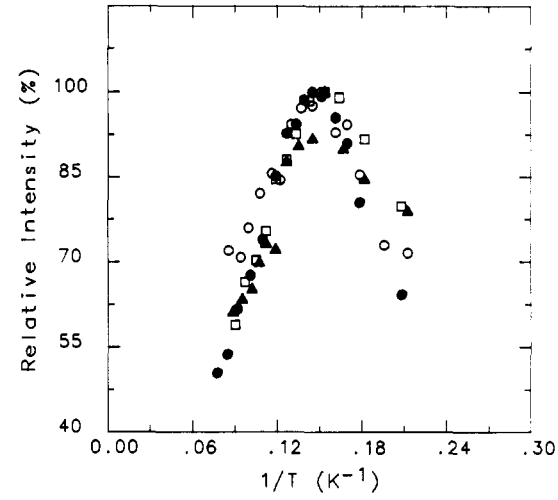


FIGURE 2: Comparison of the temperature dependences of the S_2-state EPR spectra produced in PSII membranes at pH 7.5 by illumination at 0 °C for 30 s, as shown in Figure 1. EPR spectrometer conditions and sample conditions were as described in Figure 1 except that the microwave power was set to 25 μW. The intensity of the S_2-state EPR spectrum was determined by the sum of the peak to peak heights of the hyperfine lines marked in Figure 1 below spectrum d. The maximum intensity of the S_2-state EPR spectrum observed in each type of PSII membrane preparation was normalized to 100% relative intensity: untreated PSII membranes (solid circles); PSII membranes treated with 100 mM AEPD (open circles); PSII membranes treated with 100 mM Tris (open squares); PSII membranes treated with 100 mM CH_3NH_2·HCl (solid triangles).

the addition of NH_4Cl significantly reduced the yield of the S_2-state multiline EPR signal generated at 210 K.

Temperature Dependence of the S_2-State Multiline EPR Signals. As has been previously reported, the S_2-state multiline EPR signal in untreated PSII membrane preparations arises from an excited $S = {}^1/_2$ state populated from lower lying states as the temperature increases, producing an EPR signal whose temperature dependence has a pronounced maximum at 6.9 K (de Paula & Brudvig, 1985). Figure 2 shows that the S_2-state multiline EPR signal produced by illumination at 0 °C in PSII membranes treated with AEPD, Tris, or CH_3NH_2 exhibits a temperature dependence closely following that observed in untreated samples. We previously reported (Beck et al., 1986) that the spectrum shown in Figure 1e, from NH_4Cl-treated PSII membranes illuminated at 0 °C, exhibits a temperature dependence obeying the Curie law between 5 and 25 K. Thus, NH_4Cl treatment alters both the hyperfine pattern and temperature dependence of the S_2-state multiline EPR signal, and this effect is not observed for the other amines studied.

The amine-treated PSII membrane samples used to obtain the data shown in Figures 1 and 2, however, were significantly inhibited in the presence of the amine. At 0.5 mM Cl^- and pH 7.5, PSII membranes treated with 100 mM AEPD and 100 mM Tris evolved O_2 at only 37% and 39% of the rate observed in untreated samples, respectively. In the presence of 115 mM Cl^-, PSII membranes treated with 100 mM CH_3NH_2 and 100 mM NH_4Cl at pH 7.5 evolved O_2 at 72% and 37%, respectively, of the rate observed in untreated samples.

NH_3 Binding to the S_1 and S_2 States. Our previously reported experiments with NH_4Cl-treated PSII membranes indicated that NH_3 binds to the Mn site of the OEC after formation of the S_2 state. This conclusion was based on the observation that the line shape of the S_2-state multiline EPR spectrum in the presence of 100 mM NH_4Cl is identical with that observed in untreated samples when illumination at 210

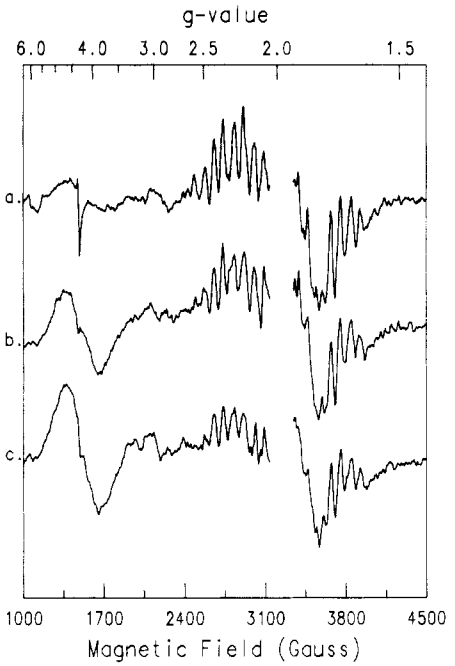


FIGURE 3: Dependence on the NH_4Cl concentration of the S_2-state EPR spectra produced in PSII membranes at pH 7.5 by illumination at 210 K for 120 s. EPR spectrometer conditions and sample conditions were as noted in Figure 1: (a) untreated PSII membranes (total $[Cl^-]$ = 15 mM); (b) PSII membranes treated with 50 mM NH_4Cl (total $[Cl^-]$ = 65 mM); (c) PSII membranes treated with 100 mM NH_4Cl (total $[Cl^-]$ = 115 mM).

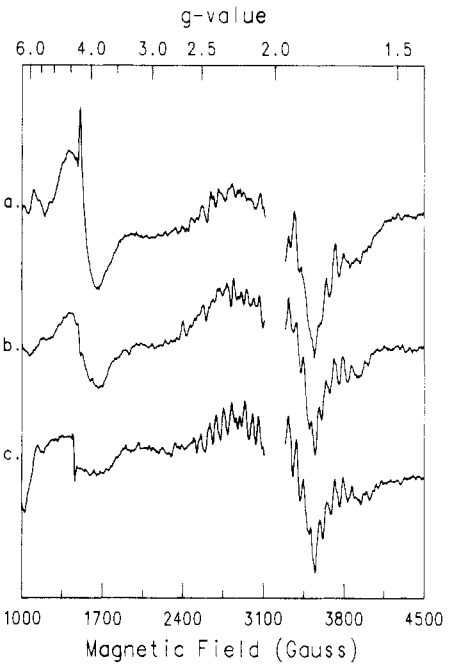


FIGURE 4: Dependence on the NH_4Cl concentration of the S_2-state EPR spectra produced in PSII membranes at pH 7.5 by illumination at 0 °C for 30 s. EPR spectrometer conditions and sample conditions were as noted in Figure 1: (a) PSII membranes treated with 10 mM NH_4Cl; (b) PSII membranes treated with 50 mM NH_4Cl; (c) PSII membranes treated with 100 mM NH_4Cl.

K is employed to produce the S_2 state. We concluded that ligand exchange must proceed slowly at 210 K relative to the rate of ligand exchange at 0 °C, since illumination at 0 °C of NH_4Cl-treated PSII membranes produces an altered S_2-state multiline EPR signal. However, it was noted that the intensity of the S_2-state multiline EPR signal observed in NH_4Cl-treated samples illuminated at 210 K was always less than that observed in untreated samples illuminated at the same temperature (Beck et al., 1986). As is shown in Figure 3, the reduced intensity of the S_2-state multiline EPR signal in NH_4Cl-treated PSII membranes illuminated at 210 K can be attributed to the production of a more stable form of the g = 4.1 EPR signal.

Figure 3a shows a wider magnetic field range of the EPR spectrum obtained after illumination of untreated PSII membranes at 210 K. The S_2-state multiline EPR signal is present, along with a small S_2-state g = 4.1 EPR signal. In untreated PSII membrane samples, the g = 4.1 EPR signal formed after illumination decays quantitatively at 210 K, forming the S_2-state multiline EPR signal in its place (de Paula et al., 1985). In contrast, the spectra in parts b and c of Figure 3 show that as the NH_4Cl concentration is increased to 50 and 100 mM, respectively, the intensity of the S_2-state multiline EPR signal decreases while the intensity of the S_2-state g = 4.1 EPR signal increases. The line width and g value of the EPR signal at g = 4.1 in the spectra shown in Figure 3b,c are indistinguishable from those previously reported for the g = 4.1 EPR signal produced in untreated PSII membranes by illumination at 130 K (Casey & Sauer, 1984; Zimmermann & Rutherford, 1984; de Paula et al., 1985). Since the intensity of the turning point at g = 1.9 of the EPR signal from $Fe^{II}Q_A^-$ is approximately the same in the spectra shown in Figure 3, the same number of charge separations at the PSII reaction center occurred in each sample during the illumination period at 210 K. Thus, the increase in the intensity of the g = 4.1 EPR signal concomitant with a decrease in the multiline EPR signal as the NH_4Cl concentration is increased is due to a stabilization of the S_2-state g = 4.1 EPR signal species relative to the S_2-state multiline EPR signal species. The results shown in Figure 3b,c are similar to the results of Zimmermann and Rutherford (1984) obtained with untreated PSII membranes in the absence of added cryoprotectant.

It is possible that at 210 K NH_3 slows down but does not prevent the normal conversion of the g = 4.1 EPR signal species to the multiline EPR signal species. To address this possibility, experiments were done by using illumination at 0 °C, a temperature at which the conversion process should proceed more rapidly. Parts a–c of Figure 4 show the S_2-state EPR spectra formed after illumination at 0 °C in the presence of 10, 50, and 100 mM NH_4Cl, respectively. No g = 4.1 EPR signal is produced in control samples in the absence of NH_4Cl (spectrum not shown), and the S_2-state multiline EPR signal observed in this case is identical with that produced by illumination at 210 K. In the presence of only 10 mM NH_4Cl, a large $Fe^{II}Q_A^-$ EPR signal was formed but only a very small S_2-state multiline EPR signal is observed. Instead, a large g = 4.1 EPR signal is produced; this EPR signal is also indistinguishable from the g = 4.1 EPR signal produced by illumination at 130 K in untreated samples. The large intensity of the g = 4.1 EPR signal in Figure 4a accounts for most of the S_2 state produced during the illumination period. The spectra in Figure 4b,c show that as the NH_4Cl concentration is increased further, the size of the g = 4.1 EPR signal is reduced as the intensity of the 67.5-G hyperfine line spacing S_2-state multiline EPR signal increases. The general trend observed in the spectra shown in Figure 4 and in spectra not shown of the same type, employing intermediate NH_4Cl concentrations, suggests that the sum of the number of spins observed in the g = 4.1 and the two multiline forms of the S_2-state EPR signal remains constant and independent of the NH_4Cl concentration.

Perhaps the most remarkable result presented in Figure 4 is that only 10 mM NH_4Cl causes a nearly complete con-

version of the multiline EPR signal species into the $g = 4.1$ EPR signal species. This NH_4Cl concentration is insufficient to cause significant inhibition of O_2 evolution activity. In fact, the data of Sandusky and Yocum (1983) and our results (data not shown), show that only about 10% inhibition occurs in the presence of 10 mM NH_4Cl. Nevertheless, the lack of a significant amount of the S_2-state multiline signal in Figure 4a shows that the majority of the sites are affected by the presence of 10 mM NH_4Cl. The results of Figure 4 confirm that NH_3 stabilizes the form of the OEC giving rise to the S_2-state $g = 4.1$ EPR signal.

The stabilization of the S_2-state $g = 4.1$ EPR signal species by NH_3 could be explained in two ways. One possibility is that NH_3 stabilizes a conformation of the OEC giving rise to the $g = 4.1$ EPR signal. In this case, both the $g = 4.1$ and multiline EPR signals would arise from the same site. Another possibility is that NH_3 blocks electron transfer between two sites, one giving rise to the $g = 4.1$ EPR signal and the other exhibiting the multiline EPR signal. In order to distinguish between these two possibilites, we performed a series of experiments employing illumination at low temperatures followed by warming to higher temperatures, following the production and decay of the multiline and $g = 4.1$ EPR signals in the S_2 state. These experiments allowed the effects of electron transfer to be distinguished from the effects of ligand exchange. Similar experiments were used previously by de Paula et al. (1985) in order to follow the path of electron donation in untreated PSII membranes.

At very low temperatures, ligand-exchange reactions proceed slowly in comparison to the rate of electron-transfer reactions. One might expect, then, that the addition of NH_3 to dark-adapted PSII membrane samples would not have any effect on the course of electron transfer on the donor side of PSII at low temperature unless ligand exchange had occurred in the dark-stable S_1 state. The spectrum in Figure 5a shows that PSII membranes treated with 10 mM NH_4Cl exhibit a $g = 4.1$ EPR signal when illuminated at 130 K for 10 min. A significant amount of cytochrome b_{559} was also photooxidized at 130 K, producing an EPR signal with turning points at $g = 3.0$ and $g = 2.2$. At this temperature cytochrome b_{559} competes with the S_1 state for electron donation to $P680^+$, and, therefore, the maximum yield of the S_2 state is only about half of that observed after illumination at 210 K (de Paula et al., 1985). Figure 5b shows the effect of warming the same sample used in Figure 5a to 210 K in the dark for 1 min. The $g = 4.1$ EPR signal observed in Figure 5a has disappeared, and a significant amount of the S_2-state multiline EPR signal has formed in its place; however, the cytochrome b_{559} EPR signal remains after the period of dark incubation at 210 K. The results of Figure 5a,b are identical with those observed previously for untreated PSII membranes (Casey & Sauer, 1984; de Paula et al., 1985), demonstrating that the addition of 10 mM NH_4Cl does not perturb the path of electron transfer in PSII membranes at temperatures below 210 K. At the higher NH_4Cl concentrations employed in Figure 3b,c, however, the formation of a more stable $g = 4.1$ EPR signal after illumination at 210 K is evidence that NH_3 does bind to the S_1 state.

Ligand-exchange chemistry also evidently occurs upon forming the S_2 state in the presence of 10 mM NH_4Cl since illumination at 0 °C forms a substantially larger amount of the S_2-state $g = 4.1$ EPR signal than does illumination at 210 K. To follow the NH_3-binding chemistry that occurs after formation of the S_2 state, we illuminated PSII membrane samples at 210 K to generate the S_2 state in the absence of rapid ligand-exchange reactions and then warmed the sample to 0 °C to allow ligand-exchange reactions to proceed. DCBQ (250 μM) was present in these PSII membrane samples to allow reoxidation of Q_A upon warming to 0 °C, increasing the lifetime of the S_2 state. Untreated PSII membranes form a large S_2-state multiline EPR signal and very little, if any, $g = 4.1$ EPR signal after illumination at 210 K, as is shown in Figure 6a. Warming the sample used in Figure 6a to 0 °C caused the $g = 1.9$ EPR signal from $Fe^{II}Q_A^-$ to completely collapse, as is observed in Figure 6b; however, only a small amount of the S_2-state multiline EPR signal decayed during the dark incubation at 0 °C. This same procedure was applied to follow the ligand-exchange reactions that occur in the S_2 state in the presence of 10 or 100 mM NH_4Cl.

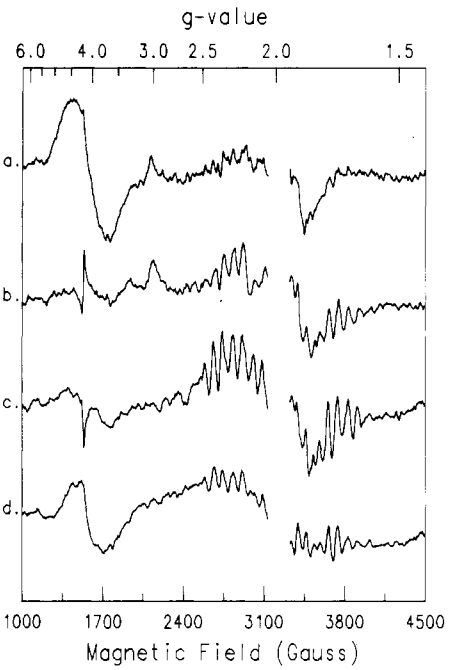


FIGURE 5: S_2-state EPR signals produced in PSII membranes treated with 10 mM NH_4Cl at pH 7.5, showing the effects of warming after illumination on the nature of the spectra observed. EPR spectrometer and sample conditions were as noted in Figure 1 except that the sample for spectra c and d contained 250 μM DCBQ: (a) PSII membranes illuminated for 10 min at 130 K followed by rapid cooling to 77 K; (b) same sample used for spectrum a then warmed to 210 K for 1 min in darkness followed by cooling to 77 K; (c) PSII membranes illuminated at 210 K for 2 min followed by cooling to 77 K; (d) same sample used in for spectrum c warmed to 0 °C for 1 min followed by rapid freezing to 77 K.

Figure 5c shows that PSII membranes treated with 10 mM NH_4Cl exhibit only a very small $g = 4.1$ EPR signal and a large S_2-state multiline EPR signal when illuminated at 210 K. The $g = 4.1$ EPR signal in Figure 5c, in fact, is about the same size as that observed in a similar experiment using untreated PSII membranes (Figure 3a). When the same sample used for the spectrum in Figure 5c was warmed to 0 °C in darkness for 1 min, a dramatic increase in the intensity of the $g = 4.1$ EPR signal occurred at the same time as a large decrease in the intensity of the S_2 state multiline EPR signal. This procedure produced the same S_2-state EPR signals as produced by illumination at 0 °C; the only significant difference between the EPR spectra in Figures 4a and 5c is the elimination of the $Fe^{II}Q_A^-$ EPR signal upon warming in the presence of excess DCBQ. It is evident that the increase in the size of the $g = 4.1$ EPR signal upon warming of the 10 mM NH_4Cl-treated sample illuminated at 210 K is due to enhanced binding of NH_3 to a site on the OEC in the S_2 state.

Effects of NH_3-binding to the OEC in the S_2 state were also

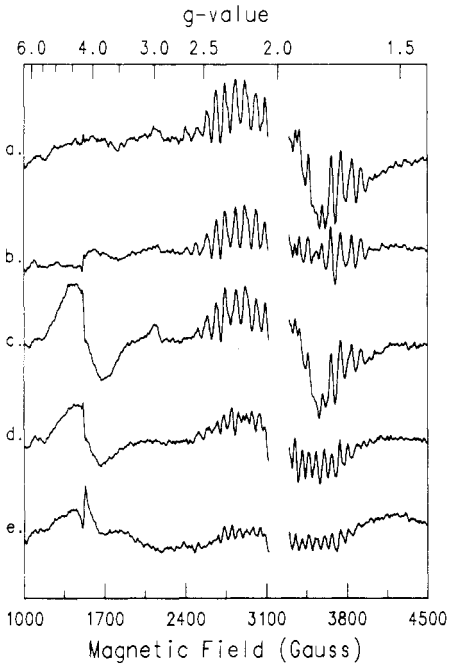
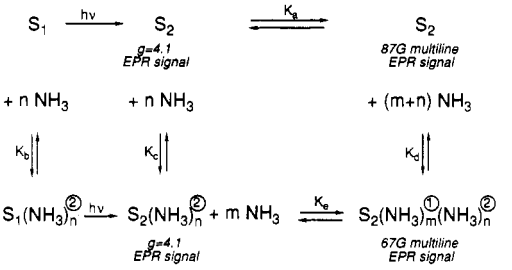


FIGURE 6: S_2-state EPR signals produced in untreated PSII membranes and in PSII membranes treated with 100 mM NH_4Cl at pH 7.5, showing the effects of warming after illumination on the spectra. EPR spectrometer conditions were as described in Figure 1 except that the samples contained 250 μM DCBQ: (a) untreated PSII membranes, illuminated at 210 K for 120 s; (b) same sample used for spectrum a then warmed to 0 °C for 1 min in darkness followed by cooling to 77 K; (c) PSII membranes treated with 100 mM NH_4Cl, illuminated at 210 K for 120 s; (d) the same sample used for spectrum c warmed to 0 °C for 1 min in darkness followed by cooling to 77 K; (e) sample used for spectra c and d warmed again to 0 °C for 20 min in the dark followed by cooling to 77 K.

observed upon warming of 100 mM NH_4Cl-treated PSII membranes after illumination at 210 K. As was discussed above, 100 mM NH_4Cl-treated PSII membranes exhibit a large $g = 4.1$ EPR signal and a reduced-intensity S_2-state multiline EPR signal after illumination at 210 K (Figure 6c). Figure 6d shows that warming the same sample used in Figure 6c to 0 °C for 1 min in darkness caused the $g = 4.1$ EPR signal to partially collapse, while at the same time the S_2-state multiline EPR signal is transformed into the 67.5-G hyperfine line spacing form. This change in the multiline EPR signal shows that NH_3 has bound directly to the Mn site.

Further incubation in the dark at 0 °C of the same 100 mM NH_4Cl-treated sample used in Figure 6c,d caused a further decrease in the intensity of the $g = 4.1$ EPR signal. In fact, the spectrum shown in Figure 6e demonstrates that even after 21 min in the dark at 0 °C, the S_2-state multiline EPR signal with 67.5-G hyperfine line spacing is still apparent while the $g = 4.1$ EPR signal has almost completely collapsed. The results shown in Figure 6e suggest that the S_2-state lifetime is quite long in the presence of NH_4Cl, as was previously reported by Velthuys (1975) and by Delrieu (1976). After additional dark incubation, reillumination at 210 K of a similar 100 mM NH_4Cl-treated sample resulted in formation of an S_2-state EPR spectrum (not shown) nearly identical with that originally produced by illumination of a dark-adapted sample at 210 K (see Figure 6c). This result shows that the loss of intensity of the 67.5-G hyperfine line spacing S_2-state multiline EPR signal upon dark adaptation returns the OEC to the same configuration and oxidation state (S_1) present before illumination. Thus, the binding of NH_3 to the Mn site in the S_2 state is readily reversible upon back-reaction to the S_1 state. The reversibility of NH_3 inhibition of photosynthetic O_2 evolution

Scheme I: Model for NH_3-Binding Equilibria in S_1 and S_2 States[a]



[a] Binding of NH_3 occurs to two different sites in the OEC, types 1 and 2, which are noted in the scheme by circled superscripts.

activity has been noted in several reports (Izawa et al., 1969; Yocum & Babcock, 1981).

Although the $g = 4$ magnetic field range is not shown in the EPR spectra of Figure 1, we looked for formation of the S_2-state $g = 4.1$ EPR signal after illumination of PSII membranes treated with AEPD, Tris, or CH_3NH_2·HCl. Under the conditions used in Figure 1a–d (and over the entire range of amine concentrations covered; data not shown), none of these samples produced a $g = 4.1$ EPR signal after illumination at 0 °C or at 210 K. Apparently, the stabilization of the $g = 4.1$ EPR species occurs only in the presence of NH_3 and not in the presence of the other amines studied.

DISCUSSION

Our previous study on the binding of NH_3 to the OEC (Beck et al., 1986) demonstrated that the S_2-state multiline EPR signal could be used to detect ligand-exchange chemistry at the Mn site. After dark-adapted PSII membranes have been illuminated at 210 K, the Mn site is left trapped in a configuration like that present in the dark-stable S_1 state despite the fact that photooxidation of the Mn site to the S_2 state has occurred. Ligand-exchange reactions that occurred in the dark S_1 state before the sample was frozen can thus be monitored when the S_2 state is produced at 210 K. Generation of the S_2 state using illumination at 0 °C allows ligand-exchange reactions to occur after the S_2 state is formed, permitting the results of ligand exchange in the S_2 state to be detected. Using these techniques, we have shown in this paper that the structure of the Mn site is affected by the binding of NH_3 to the OEC in both the S_1 and S_2 states; however, amines larger than NH_3 fail to bind to the OEC in a manner that affects the structure of the Mn site in either the S_1 or S_2 states, apparently owing to steric constraints.

Binding of NH_3 to the OEC. While low concentrations of NH_3 cause alterations in the behavior of the S_2-state $g = 4.1$ EPR signal, higher concentrations alter the hyperfine line spacing and temperature dependence of the S_2-state multiline EPR signal. Under the conditions of our experiments, the $g = 4.1$ EPR signal is unstable at temperatures above about 150 K in untreated samples (Casey & Sauer, 1984; de Paula et al., 1985), but in the presence of 100 mM NH_4Cl a large $g = 4.1$ EPR signal is observed after illumination at 210 K. As the concentration of NH_4Cl is increased, the yield of the $g = 4.1$ EPR signal increases and the yield of the multiline EPR signal decreases. These phenomena suggest a set of NH_3-binding equilibria, which are shown in Scheme I. The binding of NH_3 to both the S_1 and S_2 states influences the relative yields of the $g = 4.1$ and multiline EPR signals observed in the S_2 state.

Several points regarding the interaction of NH_3 with the OEC are noteworthy. First, changes in the yield of the $g = 4.1$ EPR signal relative to the yield of the multiline EPR signal

as the NH_4Cl concentration was increased were observed in samples in which the S_2 state was generated at 210 K (Figure 3), a temperature at which ligand exchange proceeds slowly. Thus, NH_3 must have bound to the OEC in the S_1 state. The results shown in Figure 4c, however, indicate that NH_3 can also bind in the S_2 state with a substantially increased equilibrium constant; when the S_2 state is produced by using illumination at 0 °C, only 10 mM NH_4Cl is required to nearly quantitatively produce the $g = 4.1$ EPR signal in lieu of the multiline EPR signal. Thus, the equilibrium constant K_c must be larger than the equilibrium constant K_b in the system described by Scheme I. Second, the concentration of NH_4Cl required to produce a substantial fraction of the S_2-state $g = 4.1$ EPR signal species is much lower than the concentration of NH_4Cl required to produce the altered S_2-state multiline EPR signal.

From a consideration of these points, we conclude that two types of NH_3-binding sites exist in the OEC that affect the structure of the Mn site in the S_2 state. There is evidence for a NH_3-binding site (type 1), accessible only in the S_2 state, on the Mn site itself. Binding of NH_3 to the type 1 site involves direct ligation of NH_3 to Mn in the OEC, as was argued previously on the basis of the alteration of the S_2-state multiline EPR signal and its temperature dependence (Beck et al., 1986). The type 1 binding site may be the substrate-binding site of the OEC because the concentration of NH_4Cl needed to saturate this site is comparable to the concentration of NH_4Cl at which O_2 evolution is inhibited. Binding of NH_3 to a second type of binding site (type 2) in the OEC, but not necessarily on the Mn site, affects the stability of the $g = 4.1$ EPR signal. The type 2 NH_3-binding site exchanges ligands in the S_1 state as well as in the S_2 state, although NH_3 binds more strongly in the S_2 state. The $g = 4.1$ EPR signal present when NH_3 is bound to the type 2 site has exactly the same line width and g value as the $g = 4.1$ EPR signal observed after 130 K illumination of untreated PSII membranes. In view of this observation and our conclusion that the $g = 4.1$ and multiline EPR signals arise from the same paramagnetic site (see below), it seems less likely that the type 2 NH_3-binding site is on Mn.

Binding of Amines Other Than NH_3 to the OEC. The hyperfine line pattern and temperature dependence of the S_2-state multiline EPR signal were unaffected by the presence of the amines AEPD, Tris, and CH_3NH_2; identical spectra were obtained when illumination at either 210 K or 0 °C was employed to generate the S_2 state. These results provide spectroscopic evidence against the ligation of amines larger than NH_3 to the Mn site in the S_2 state. In addition, we did not observe alterations of the low-temperature behavior of the $g = 4.1$ EPR signal in PSII membranes treated with amines other than NH_3. Hence, amines larger than NH_3 also fail to bind to the type 2 binding site in either the S_1 or the S_2 states under the conditions used.

The question remains concerning the location of the binding site responsible for reversible amine inhibition in the OEC. It is possible that amines other than NH_3 can only bind to the Mn site in the S_3 state and, in so doing, inhibit O_2 evolution. If this is the case, then EPR studies of ligand substitution reactions in the S_1 and S_2 states would not reveal changes in the Mn site in the presence of these amines. Another possibility is suggested by the experiments of Sandusky and Yocum (1984, 1986), which showed that the inhibition of O_2 evolution by amines depends on the Cl^- concentration. It was concluded that Cl^-, an essential cofactor for O_2 evolution (Kelley & Izawa, 1978), is displaced by amines and by F^- (Sandusky & Yocum, 1986). Several studies have indicated that Cl^- is bound to the OEC in the S_2 state (Theg et al., 1984; Itoh et al., 1984; Preston & Pace, 1985). If Cl^- is, in fact, bound to the OEC in the S_2 state and if Cl^- is displaced by amines, then our results indicate that the Cl^-/amine-binding site is not on the Mn site.

The results of Frasch and Cheniae (1980) indicate that Tris irreversibly inhibits O_2 evolution activity in illuminated spinach thylakoid membranes by a specific binding to the S_2 state, which results in displacement of Mn ions from the OEC. One explanation for their observations involves direct binding of Tris to the Mn site, eventually causing disruption and displacement of Mn ions. Our failure to observe evidence for binding of Tris to the Mn site in the S_2 state may be accounted for if our conditions permit only reversible ligand binding to the Mn site; it is possible that at the higher pH and higher Tris concentrations employed in the studies of Frasch and Cheniae (1980) one or more Tris molecules bind to the Mn site and cause a subsequent release of Mn ions. The finding that NH_3 protects against Tris inactivation of O_2 evolution activity (Frasch & Cheniae, 1980) is consistent with our observation that NH_3 does bind to the Mn site in the S_2 state; the presence of NH_3 bound to the Mn site may prevent the binding of Tris and associated loss of O_2 evolution activity.

Origin of the $g = 4.1$ and Multiline EPR Signals. The experiments reported in this paper reveal new aspects of the properties of the S_2-state $g = 4.1$ EPR signal. Casey and Sauer (1984) attributed the $g = 4.1$ EPR signal to a non-heme Fe(III) functioning as an electron carrier between the OEC and $P680^+$. Based on independent experiments, Zimmermann and Rutherford (1984) also attributed the $g = 4.1$ EPR signal to an intermediate electron donor between the Mn site and the primary electron donor to the PSII reaction center. More recently, it was proposed that the $g = 4.1$ EPR signal and the multiline EPR signal observed in the S_2 state arise from different configurations of the same Mn-containing paramagnetic site (de Paula et al., 1985). The results of theoretical studies provided support for the conclusion that a single paramagnetic site accounts for both the $g = 4.1$ and multiline EPR signals (de Paula et al., 1986).

The electron carrier model of Casey and Sauer (1984) and of Zimmermann and Rutherford (1984) accounts for the $g = 4.1$ and multiline EPR spectra by proposing that two distinct paramagnetic sites exist on the electron donor side of PSII. Such a model fails to account for the observation by de Paula et al. (1985) that the $g = 4.1$ EPR signal is produced only in the S_2 state. Additionally, the behavior of the paramagnetic site exhibiting the $g = 4.1$ EPR signal in PSII membranes treated with 10 mM NH_4Cl is not consistent wth the behavior of an electron carrier without invoking reversal of electron transfer upon binding of NH_3. Consider the results obtained from a sample treated with 10 mM NH_4Cl. Illumination at 130 K produces a $g = 4.1$ EPR signal. Warming this sample to 210 K causes a conversion of the $g = 4.1$ EPR signal into the multiline EPR signal. Further warming of this sample to 0 °C regenerates the $g = 4.1$ EPR signal with loss of the multiline EPR signal. One could argue that the relative redox potentials of the two hypothetical paramagnetic sites from which the $g = 4.1$ and multiline EPR signals arise are reversed when NH_3 binds after the sample is warmed to 0 °C. Such an argument, however, seems less probable considering the opposite behavior observed in the presence of still higher NH_4Cl concentrations, which yields another multiline EPR signal having a 67.5-G hyperfine line spacing.

Thus, the results of this paper provide strong support for the proposal that the $g = 4.1$ and multiline EPR signals arise

from the same paramagnetic site in two different configurations. We can further conclude that these two configurations are linked by an equilibrium influenced by the binding of NH_3 to the OEC and, perhaps other factors as well (see below). In untreated PSII membranes, the size of the equilibrium constant K_a in Scheme I, describing the ratio of the intensities of the $g = 4.1$ and multiline EPR signals observed in the S_2 state, must be much larger than 1 to account for the quantitative decay of the $g = 4.1$ EPR signal upon warming to high temperatures. Binding of NH_3 to the type 2 binding site apparently shifts the equilibrium between the two forms to favor the configuration exhibiting the $g = 4.1$ EPR signal. At higher concentrations of NH_4Cl, NH_3 binds to the type 1 site, producing the form of the S_2 state exhibiting the multiline EPR signal having a 67.5-G hyperfine line spacing. This model is consistent with the observation that the S_2-state $g = 4.1$ EPR signal produced when the type 2 NH_3-binding site is saturated is indistinguishable from that observed in untreated samples.

The proposal that the $g = 4.1$ and multiline EPR signals observed in the S_2 state arise from two different configurations of a single paramagnetic site is consistent with the behavior of the $g = 4.1$ EPR signal reported by Casey and Sauer (1984) in the presence of F^- and with the results obtained by Zimmermann and Rutherford (1984) in the absence of cryoprotectant. Casey and Sauer (1984) found that F^- stabilized the $g = 4.1$ EPR signal produced by illumination of PSII membranes at 200 K in a manner similar to that observed in the presence of NH_4Cl. It seems likely that F^- binds to the type 2 NH_3-binding site and, in so doing, stabilizes the $g = 4.1$ EPR signal form of the S_2 state. Zimmermann and Rutherford (1984) found that a significant yield of the $g = 4.1$ EPR signal was obtained in the absence of glycerol after dark-adapted untreated PSII membranes were illuminated at 200 K. This result can be accounted for if the solution conditions cause a shift of the equilibrium between the forms of the Mn site exhibiting the $g = 4.1$ and multiline EPR signals in the S_2 state.

Conclusions

The results of this paper support the conclusions of Beck et al. (1986) that a single, Mn-containing site exists on the electron donor side of PSII that functions both in storage of oxidizing equivalents and in binding and oxidation of substrate H_2O molecules. NH_3 binds directly to the Mn site in the S_2 state; bulkier amines such as Tris, AEPD, and even CH_3NH_2 apparently cannot bind to the Mn site owing to steric factors. An additional NH_3-binding site exists in the OEC, but not necessarily on the Mn site, and influences a equilibrium between the forms of the Mn site from which the $g = 4.1$ and multiline EPR signals arise. This second NH_3-binding site also has steric selectivity for small Lewis bases since amines larger than NH_3 did not alter the properties of the $g = 4.1$ EPR signal under the conditions used.

Registry No. NH_4Cl, 12125-02-9; NH_3, 7664-41-7.

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