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The Manganese Site of the Photosynthetic Oxygen-Evolving Complex Probed by EPR Spectroscopy of Oriented Photosystem II Membranes: The $g = 4$ and $g = 2$ Multiline Signals†

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ABSTRACT: The $g = 4$ and $g = 2$ multiline EPR signals arising from the Mn cluster of the photosynthetic oxygen-evolving complex (OEC) in the S_2 state were studied in preparations of oriented photosystem II (PSII) membranes. The ammonia-modified forms of these two signals were also examined. The $g = 4$ signal obtained in oriented PSII membranes treated with NH_4Cl at pH 7.5 displays at least 16 partially resolved Mn hyperfine transitions with a regular spacing of 36 G [Kim, D. H., Britt, R. D., Klein, M. P., & Sauer, K. (1990) *J. Am. Chem. Soc. 112*, 9389-9391]. The observation of this $g = 4$ "multiline signal" provides strong spectral evidence for a tetranuclear Mn origin for the $g = 4$ signal and is strongly suggestive of a model in which different spin state configurations of a single exchange-coupled Mn cluster give rise to the $g = 4$ and $g = 2$ multiline signals. A simulation shows the observed spectrum to be consistent with an $S = 3/2$ or $S = 5/2$ state of a tetranuclear Mn complex. The resolution of hyperfine structure on the NH_3-modified $g = 4$ signal is strongly dependent on sample orientation, with no resolved hyperfine structure when the membrane normal is oriented perpendicular to the applied magnetic field. The dramatic NH_3-induced changes in the $g = 4$ signal resolved in the spectra of oriented samples are suggestive that NH_3 binding at the Cl^- site of the OEC may represent direct coordination of NH_3 to the Mn cluster. Orientation dependence data on the $g = 2$ multiline signal show that the hyperfine transitions in the wings of the signal are anisotropic. These peaks remain unresolved in the powder spectrum of the $g = 2$ multiline signal. The g tensor for the $g = 2$ multiline signal may be more anisotropic than previously thought.

The process of plant and cyanobacterial photosynthesis is coupled to the oxidation of water by the photosystem II (PSII)[1] reaction center complex. Four consecutive photoinduced one-electron charge separations in the PSII reaction center core are coupled to the four-electron oxidation of water by a Mn-containing enzyme, the oxygen-evolving complex (OEC). The OEC cycles through five intermediate catalytic states, designated S_0 through S_4; the conversion of S_4 to S_0 is accompanied by the release of O_2 (Kok et al., 1970). Four Mn atoms are associated with each PSII reaction center (Yocum et al., 1981) and form the active site of the OEC. The structure of the Mn site and the roles of the cofactors Ca^{2+} and Cl^-, both essential for oxygen evolution activity, have been a topic of intensive investigation [reviewed in Rutherford (1989), Sauer et al. (1991), and Debus (1991)].

Electron paramagnetic resonance (EPR) spectroscopy has been a powerful method for probing the structure of the OEC. Two EPR signals have been assigned to the Mn site of the OEC in the S_2 state. The $g = 2$ multiline signal displays at least 18 well-resolved Mn hyperfine transitions, providing direct spectroscopic evidence for the existence of an exchange-coupled Mn cluster of at least two Mn atoms (Dismukes & Siderer, 1981; Hansson & Andréasson, 1982). The second signal associated with the Mn site of the OEC in the S_2 state is centered at $g \approx 4$, has a peak-to-peak width of 300-400 G, and lacks resolved hyperfine features in PSII membranes prepared without ammonia treatment (Casey & Sauer, 1984; Zimmermann & Rutherford, 1984, 1986; Cole et al., 1987). We recently reported the observation of at least 16 Mn hyperfine transitions with a regular spacing of 36 G on the $g =$

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[1] Abbreviations: PSII, photosystem II; OEC, oxygen-evolving complex; EPR, electron paramagnetic resonance; ESEEM, electron spin-echo envelope modulation; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Chl, chlorophyll; PPBQ, phenyl-*p*-benzoquinone; Q_A, primary quinone electron acceptor in PSII.

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4 EPR signal obtained from oriented NH_3-treated PSII preparations (Kim et al., 1990). This observation allowed us to make a conclusive assignment of the $g = 4$ signal to a multinuclear Mn cluster, ruling out previous models for the Mn site of the OEC that involved a mononuclear Mn(IV) origin for the $g = 4$ signal (Hansson et al., 1987; Pecoraro, 1988; Penner-Hahn et al., 1990). Our results provided strong support for a model (dePaula et al., 1985; Zimmermann & Rutherford, 1986; Beck & Brudvig, 1986; Brudvig, 1989) in which the $g = 4$ and $g = 2$ multiline signals arise from different spin state configurations of an exchange-coupled tetranuclear Mn cluster.

In addition to providing information regarding the nuclearity of the Mn cluster of the OEC, studies of the Mn EPR signals have also revealed information regarding ligand-exchange chemistry occurring in the OEC, because the EPR spectra are sensitive to changes in the local environment of the Mn site. In particular, ammonia has been a useful spectroscopic probe of the Mn site because of the changes in the S_2 state Mn EPR signals induced by the binding of NH_3 to two distinct sites in the OEC. NH_3 binding to a Cl^--sensitive site in the S_1 and S_2 states leads to the stabilization of the $g = 4$ signal relative to the $g = 2$ multiline signal (Beck & Brudvig, 1986; Andréasson et al., 1988). This NH_3-binding site probably is the binding site for Cl^- in at least the S_1 state of the OEC, and it has been speculated that water binding to this site may occur in later S states (Boussac et al., 1990). However, it is not clear whether binding at this site represents direct ligation to the Mn cluster or binding to a site in close proximity to the Mn cluster (Beck & Brudvig, 1986, 1988).

NH_3 also binds to a second site (independent of Cl^- concentration) in the S_2 state of the OEC, which results in an "altered" $g = 2$ multiline signal with the distinct EPR signature of a reduction in hyperfine line spacing from 87.5 to 67.5 (Beck et al., 1986). Beck et al. (1986) proposed that the change in the EPR spectrum was due to direct coordination of NH_3 to the Mn cluster at this non-Cl^- site. The results of an electron spin-echo envelope modulation (ESEEM) spectroscopy study on the ammonia-altered $g = 2$ multiline signal conclusively demonstrated that NH_3 binding at this site in fact represents direct coordination of an NH_3-derived ligand to the Mn complex (Britt et al., 1989).

We have used EPR spectroscopy to study the structural and magnetic properties of the Mn cluster of the OEC in oriented PSII membranes, both with and without ammonia treatment. EPR studies of oriented membrane systems [described in Blum et al. (1978)] may provide greater resolution of spectral features than those of nonoriented samples. In nonoriented samples a "powder pattern" spectrum results from the average over all orientations of the molecular axes with respect to the applied magnetic field. Anisotropic spectral components may remain unresolved owing to the superposition of overlapping EPR transitions or the effects of anisotropic line broadening present in the powder spectrum. In these cases the analysis of oriented membrane systems may provide a partial separation of transitions arising from different components of the g and/or hyperfine tensors, effectively reducing the anisotropy in the spectrum and leading to greater resolution. For a particular transition along a single component of the g tensor, the fortuitous alignment of this component either perpendicular to or (especially) parallel to the membrane normal (the axis of orientation) will allow considerably better resolution of the transition in the oriented sample than in a nonoriented sample. In addition, the study of oriented membrane systems may give information regarding the orientation of the magnetic axes of a paramagnetic center with respect to the membrane normal.

In this paper, we present the native and ammonia-modified forms of the $g = 4$ and $g = 2$ multiline signals observed in oriented PSII membranes. We have been able to obtain information regarding the degree of anisotropy present in the signals as well as to observe new features that have remained unresolved in previous EPR studies on nonoriented PSII preparations. In a preliminary communication, we reported the observation of a $g = 4$ multiline signal obtained from oriented NH_3-treated preparations (Kim et al., 1990). In the present paper, we present the orientation dependence of this signal as well as a discussion and simulation of the spectrum based on a tetranuclear Mn cluster as the origin for the $g = 4$ signal. Also, in the oriented spectra, we are able to resolve dramatic changes in the $g = 4$ signal upon treatment with ammonia. The implications of these spectral changes on NH_3 binding at the Cl^- site of the OEC are discussed. The orientation dependence data of the $g = 2$ multiline signals are suggestive of some anisotropy in the g tensor of the signal. Information regarding signal anisotropy is important for a detailed understanding of the Mn EPR spectra; the data presented here should be useful in future attempts at spectral simulation and modeling of the Mn site of the OEC.

Materials and Methods

PSII membranes with and without ammonia treatment were prepared and handled as described previously (Britt et al., 1989) with the following exceptions. Sample illumination at 195 K was carried out for 5 min instead of 10 min, and annealing of the samples was performed at 0 °C for 1 min instead of 20 °C for 30 s. For experiments performed at pH 6.0, 40 mM MES was used instead of 40 mM HEPES.

PSII membrane samples were oriented using a modification of the method described by Rutherford (1985). In our procedure, a thick suspension of PSII membranes (~10 mg of Chl/mL) was painted onto Mylar films. The painted strips were then allowed to dry for 1–3 h under a flow of N_2 gas in the dark at 4 °C. Two to four strips were loaded into a quartz EPR tube, and the sample was stored in darkness at 77 K until EPR spectra were taken. Sample orientation and placement in the EPR cavity was verified by measurements of the anisotropy of EPR signals arising from the cytochrome b_{559} complex and the D^+ tyrosine radical of PSII (Rutherford, 1985). In this paper, the indicated angle in the spectra of oriented samples corresponds to the angle between the membrane normal and the applied magnetic field.

EPR spectrometer conditions were as follows: microwave frequency, 9.22 GHz; microwave power, 20 mW; field modulation amplitude, 20 G for measurements of the $g = 2$ multiline signal and 10 G for measurements of the $g = 4$ signal; sample temperature, 8 K. Dark (S_1 state) spectra have been computer subtracted from the spectra recorded after sample illumination and/or annealing.

Results

The g = 4 Spectra from Oriented PSII Membranes. Figure 1A displays the S_2 state $g = 4$ EPR signal obtained from oriented PSII membranes treated with 100 mM NaCl at pH 7.5 in the presence of 400 mM sucrose. The membrane normal is oriented parallel to the applied magnetic field. The 0° spectrum is shifted to higher field relative to the 90° (90° spectrum not shown), consistent with the results of Rutherford (1985).

Figure 1B shows the $g = 4$ EPR spectra obtained from oriented PSII membranes treated with 100 mM NH_4Cl at pH 7.5 in the presence of 400 mM sucrose. Comparison of the

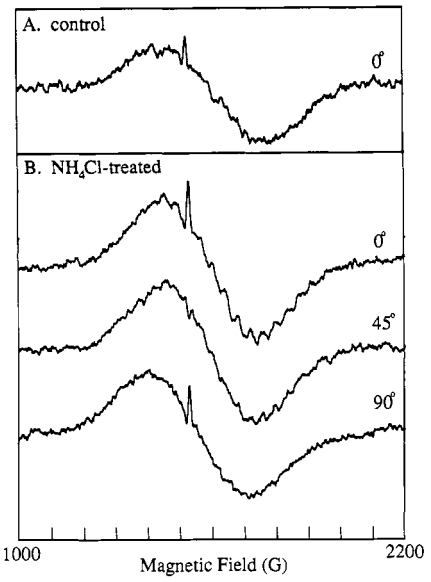

FIGURE 1: $g = 4$ EPR signal obtained from oriented PSII membranes (pH 7.5, 400 mM sucrose) illuminated at 195 K for 5 min and treated with (A) 100 mM NaCl or (B) 100 mM NH_4Cl. The indicated angle is between the membrane normal of the oriented sample and the applied magnetic field. The sharp feature at $g = 4.3$ is a subtraction artifact resulting from rhombic Fe^{3+} present in these preparations.

0° spectra in Figure 1A,B shows that the addition of ammonia induces significant changes in the $g = 4$ signal. The most important effect is the appearance of at least 16 partially resolved hyperfine transitions with a regular spacing of 36 G as reported previously (Kim et al., 1990). The resolution of hyperfine peaks is strongly dependent on sample orientation. As the angle between the membrane normal and the magnetic field is incremented from 0° to 90°, the resolution of hyperfine features is sharply diminished (Figure 1B). The resolution is also strongly dependent on degree of sample orientation. The orientation dependence data in Figure 1B also show that the signal at 90° is broader, diminished in amplitude, and shifted to lower field relative to the 0° spectrum.

The ammonia-treated preparations showed greater yield of the $g = 4$ signal relative to the $g = 2$ multiline signal, as compared to untreated control preparations. In both control and ammonia-treated PSII samples, sucrose was present as cryoprotectant. The addition of 4% ethanol to control PSII membranes led to enhancement of the $g = 2$ multiline signal at the expense of the $g = 4$ signal, consistent with previous work (Zimmermann & Rutherford, 1986). However, the yield of the $g = 4$ signal in ammonia-treated preparations was unaffected by the addition of 4% ethanol. We also note that we were unable to resolve any differences in the $g = 4$ spectra of $^{15}NH_4Cl$ as compared to the $g = 4$ spectra of $^{14}NH_4Cl$ treated samples (data not shown).

Figure 2A displays the $g = 4$ "multiline signal" obtained from another oriented ammonia-treated preparation with the membrane normal parallel to the magnetic field together with a simulation of the spectrum (Figure 2B) incorporating hyperfine couplings from four Mn atoms, as would be the case for a tetranuclear cluster origin for the $g = 4$ signal. The following isotropic parameters provided a satisfactory fit to the spectrum: $g = 4.1$; Mn hyperfine coupling constants, $|A_1| = 45$ G, $|A_2| = 37$ G, $|A_3| = 34$ G, and $|A_4| = 16$ G; Gaussian line width (half-width at half-maximum), 16 G.

Orientation Dependence of the g = 2 Multiline Signal. Figure 3 shows the orientation dependence of the $g = 2$ multiline signal obtained from PSII preparations at pH 6.0 in the presence of 400 mM sucrose and 4% ethanol after

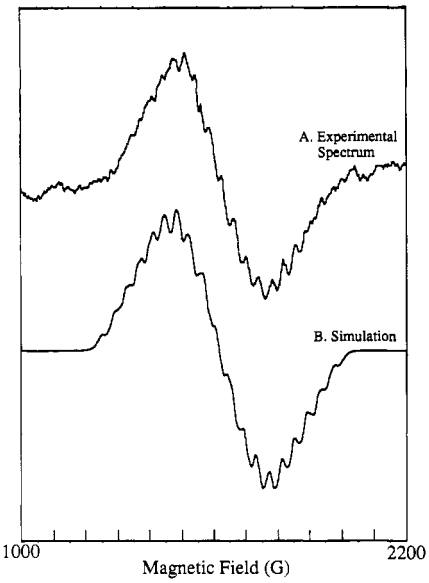

FIGURE 2: (A) $g = 4$ "multiline signal" observed from oriented ammonia-treated PSII membranes with the membrane normal oriented parallel to the applied magnetic field. The sample is different from that used for Figure 1B, 0°. (B) Simulation of the spectrum in Figure 2A utilizing an effective $S' = 1/2$ system and Gaussian line shapes. The following isotropic parameters were used: $g_{eff} = 4.1$, $\Delta_{HWHM} = 16$ G, $|A_1| = 47$ G, $|A_2| = 37$ G, $|A_3| = 34$ G, and $|A_4| = 16$ G.

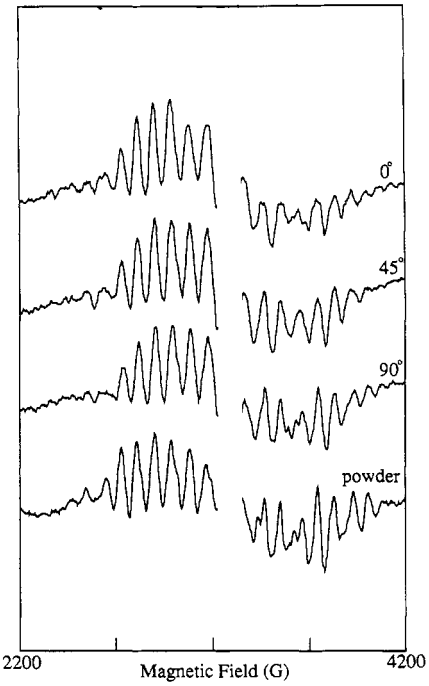

FIGURE 3: Orientation dependence of the $g = 2$ multiline signal obtained from PSII membranes (pH 6.0, 400 mM sucrose, 4% ethanol, 1 mM EDTA) illuminated at 195 K for 5 min and annealed at 0 °C for 1 min in the presence of 1 mM PPBQ. The indicated angle is between the membrane normal and the applied magnetic field for spectra recorded from oriented PSII membrane samples. The "powder" spectrum was recorded from a nonoriented sample (≈10 mg of Chl/mL). The off-scale signal due to D^+ in the $g \approx 2$ region of the spectrum has been deleted.

illumination of the sample at 195 K for 5 min followed by annealing of the sample at 0 °C for 1 min. These preparations also contained PPBQ (phenyl-*p*-benzoquinone) at a concentration of 1 mM to allow reoxidation of Q_A upon annealing of the sample. The reoxidation of Q_A stabilized the S_2 state at the annealing temperature by preventing charge recombination in the reaction center, while causing the highly aniso-

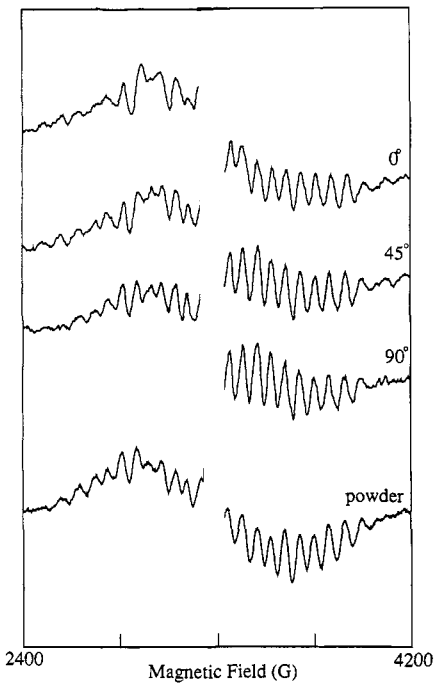

FIGURE 4: Orientation dependence of the ammonia-altered $g = 2$ multiline signal obtained from PSII membranes (100 mM NH_4Cl, pH 7.5, 400 mM sucrose, 4% ethanol, 1 mM PPBQ, 1 mM EDTA) illuminated at 195 K for 5 min and annealed at 0 °C for 1 min. The indicated angle is between the membrane normal and the applied magnetic field for spectra recorded from oriented PSII membrane samples. The "powder" spectrum was recorded from a nonoriented sample (≈10 mg of Chl/mL). The off-scale signal due to D^+ in the $g \approx 2$ region of the spectrum has been deleted.

tropic $g = 1.8$–1.9 EPR signal arising from $Fe^{2+}Q_A^-$ to disappear. This procedure allowed us to examine the orientation dependence of the high-field side ($g < 2$) of the signal.

The resolution of the lowest and highest field peaks in the wings of the $g = 2$ multiline signal in Figure 3 is greatest in the 0° spectrum. Of particular significance is the apparent lack of resolution of these extreme peaks in the powder spectrum obtained from a nonoriented preparation. This is most probably due to anisotropy in the wings of the signal, because the amplitude of the lowest field peaks decreases as the angle between the membrane normal and magnetic field is incremented from 0° to 90°, while the highest field peaks show orientation-dependent shifts in field position. The high-field region of the signal shows greater anisotropy than the low-field region, with peaks showing small orientation-dependent changes in amplitude and position. Comparison of the oriented spectra with the powder spectra obtained from nonoriented preparations shows that the powder spectrum results from the superposition of anisotropic transitions that are partially resolved in the oriented spectra. This anisotropy also contributes to increased width of some of the individual hyperfine peaks in the powder spectrum relative to those in the oriented spectra.

Figure 4 shows the orientation dependence of the NH_3-altered $g = 2$ multiline signal after illumination and annealing of the sample. The lowest and highest field peaks of the signal are resolved most clearly in the 0° and 45° spectra. The 90° spectrum shows greater amplitudes of the Mn hyperfine transitions in the central $g = 2$ region of the spectrum.

DISCUSSION

Origin of the g = 4 EPR Signal. In an earlier communication (Kim et al., 1990), we discussed the implications of the observation of the $g = 4$ multiline signal on the nuclearity of the Mn cluster of the OEC. The resolution of at least 16 Mn hyperfine transitions with a regular spacing of 36 G on the $g = 4$ signal in ammonia-treated preparations (Figure 2) provided strong support for a model in which both the $g = 4$ and $g = 2$ multiline signals arise from different spin-state configurations of an exchange-coupled tetranuclear Mn cluster (dePaula et al., 1985; Zimmermann & Rutherford, 1986; Beck & Brudvig, 1986; Brudvig, 1989). The reduced Mn hyperfine couplings observed in the $g = 4$ multiline spectrum were shown to be consistent with a tetranuclear Mn cluster origin for the signal by setting up a simplified C_{2v} Hamiltonian for a $Mn_1(III)Mn_3(IV)$ exchange-coupled tetranuclear complex and calculating the expected Mn hyperfine couplings for such a complex.

For an $S = 3/2$ state of a such a tetranuclear Mn cluster, the projected Mn hyperfine couplings were found to be 2/3, −1/3, 1/3, and 1/3 the hyperfine coupling values for the monomeric Mn ions. A similar calculation of the projected hyperfine couplings for an $S = 5/2$ state yields 2/5, −1/5, 2/5, and 2/5 the monomeric Mn value.[2] The hyperfine coupling constant for a monomeric Mn(IV) has been measured to be 75 G (Geschwind et al., 1962), while the isotropic hyperfine coupling for a monomeric Mn(III) is expected to be 80–90 G (Al'tshuler & Kozyrev, 1974).[3] Given the approximations (i.e., the assumption of C_{2v} symmetry) involved in these calculations, we consider the obtained values of the Mn hyperfine couplings to provide a qualitative illustration of the reductions in the hyperfine couplings that occur in an $S \geq 3/2$ state of a tetranuclear complex. Using values for the Mn hyperfine couplings that are consistent with the reduced values obtained from these simplified vector coupling calculations, we have performed a simulation of the $g = 4$ multiline signal. Comparison of the simulation and the experimental spectrum in Figure 2 illustrates that the simulation utilizing isotropic parameters reproduces both the hyperfine structure on the signal and the overall line shape of the experimental spectrum. The success of the simulation provides further support for a model in which the $g = 4$ signal of the S_2 state of the OEC arises from an $S \geq 3/2$ ground state of a tetranuclear Mn cluster. Implicit in this model is that the $g = 2$ multiline signal arises from an $S = 1/2$ ground state configuration of the same cluster, with the interconversion between the $g = 2$ and $g = 4$ Mn EPR signals governed by changes in the tetranuclear Mn exchange couplings that are modulated by slight structural changes (Brudvig, 1989).

We note that although Mn hyperfine structure is observed on the $g = 4$ signal only in oriented PSII membrane samples treated with ammonia, the discussion regarding the nuclearity of the Mn cluster can be extended to include the S_2 state of the OEC in PSII membranes without ammonia treatment (i.e., the native OEC). Although Figure 1 shows clear changes in the EPR spectrum induced by the binding of NH_3 to the Cl^- site of the OEC, these are minor changes compared to what would be expected if the Mn site underwent a major structural

[2] More explicitly, the Mn hyperfine projections for an $S = 5/2$ state of a $Mn_1(III)Mn_3(IV)$ complex with C_{2v} symmetry are $A_A = 2/5A_{(III)}$, $A_B = -1/5A_{(IV)}$, and $A_C = A_D = 1/5A_{(IV)}$, where $A_{(III)}$ and $A_{(IV)}$ are the hyperfine coupling constants for monomeric Mn(III) and Mn(IV), respectively. The $S = 5/2$ ground state can arise from ferromagnetic coupling between an $S_{AB} = 1/2$ ground state of an antiferromagnetically coupled $Mn_{AB}(III,IV)$ and an $S_{CD} = 2$ excited state of an antiferromagnetically coupled $Mn_{CD}(IV,IV)$.

[3] We note, however, that EPR studies of inorganic Mn(III) compounds have indicated that there may be significant anisotropy present in the hyperfine tensors of Mn(III) complexes (Dexheimer et al., 1989). This would increase the range of the expected monomeric Mn(III) hyperfine coupling to 50–110 G.

reorganization. Given the overall similarity of the native and NH_3-stabilized $g = 4$ signals, in line shape and field position as well as the behavior of the $g = 4$ signal with respect to interconversion with the $g = 2$ multiline signal (Beck & Brudvig, 1986), it is clear that the NH_3-stabilized form of the $g = 4$ signal represents a perturbed version of the native $g = 4$ signal. To be completely explicit, given a multinuclear Mn origin for the NH_3-stabilized $g = 4$ signal, it is *extremely* doubtful that the native $g = 4$ signal arises from a mononuclear Mn(IV) center.

Returning to the simulation in Figure 2, it is noteworthy that isotropic parameters provide a satisfactory account of the $g = 4$ multiline signal. In the $g = 4$ spectra obtained from oriented NH_3-treated PSII samples (Figure 1B), the sharply anisotropic resolution of hyperfine features, the reduced width of the signal, and the distinct shift to high field of the 0° signal relative to the 90° spectrum indicate that the oriented spectra display transitions arising from an angle-dependent mixture of different components of an anisotropic g tensor. In the oriented membrane system examined, only a single spectral feature can be maximum at 0°. The most likely explanation of the dramatic orientation dependence of the signal is the approximate alignment of a single principal component of the g tensor along the membrane normal, in which case a single g tensor component would make the major contributions to the 0° spectrum. The 90° spectrum would then arise from transitions due to a second component at lower field (which would account for the shift in the signal to low field) and possibly a third component also oriented in the plane of the membrane.

The $g = 4$ signal has been proposed to arise from an axially distorted $S = 3/2$ system with $D \gg h\nu$ (Hansson et al., 1987). For such a system, the principal components of the g tensor give effective g values of 4, 4, and 2 (Weltner, 1983). While a slight deviation from axial symmetry may produce a splitting in the low-field components, it is unclear how such an origin can account for the orientation dependence data in Figure 1B. In particular, in the case where the 0° spectrum arises from a single $g \approx 4$ component, the 90° spectrum would then arise from transitions from $g \approx 4$ and $g \approx 2$ principal components. However, the $g = 4$ region of the 90° spectrum would be expected to have a considerably more absorption-like even character than the symmetric derivative-shaped line that is observed in Figure 1B. In addition, we would expect a significant attenuation of this $g \approx 4$ component at 90° relative to the $g = 4$ signal at the 0° orientation due to the averaging of the $g \approx 4$ component of the 90° spectrum arising from the random orientation of the membranes in the plane of the Mylar films. The decrease in amplitude of the signal observed at 90° as compared to the 0° signal appears to be compensated by the increased width of the signal observed at 90°.

On the basis of multifrequency EPR studies, Haddy et al. (1991) have recently proposed that the $g = 4$ signal arises from a rhombically distorted ($E/D \approx 0.25$) $S = 5/2$ system with $D \approx h\nu$. The middle Kramers doublet of such a spin system should give rise to $g \approx 4$ transitions for all three principal directions of the g tensor axes (Aasa, 1970). Within the framework of this model, the orientation data in Figure 1B may be explained in a more straightforward manner. The 0° spectrum most likely consists of contributions from a single component of the g tensor. The superposition of two $g \approx 4$ resonances (from the other two axes of the g tensor) would broaden the hyperfine structure as well as account for the increased line width observed in the 90° spectrum. The orientation dependence data of the NH_3-modified $g = 4$ signal appear to be most consistent with the $S = 5/2$ origin proposed by Haddy et al., rather than with an axially distorted $S = 3/2$ origin. In either case, the lack of resolved hyperfine features in the NH_3-modified signal at the 90° orientation is most probably due to increased anisotropy resulting from the superposition of an additional g tensor component in the plane of the membrane.

Effect of the Binding of NH_3 to the Cl^- Site of the OEC. Two effects can be attributed to the binding of NH_3 to the Cl^- site of the OEC: (1) stabilization of the $g = 4$ EPR signal conformation of the Mn cluster relative to the $g = 2$ multiline signal even in the presence of 4% ethanol, and (2) alteration in the $g = 4$ signal, including the induction of resolved Mn hyperfine features in the 0° oriented spectrum (Figure 1). The observed stabilization of the $g = 4$ signal is fully consistent with the results of Beck and Brudvig (1986), who used ethylene glycol as cryoprotectant, and Andréasson and Hansson (1988), who used sucrose as cryoprotectant. Our experiments were performed in the presence of sucrose. However, we found that the NH_3-modified $g = 4$ signal was unaffected by the addition of 4% ethanol. The behavior of the Mn EPR signals in the presence of ethylene glycol is known to be similar to the behavior in sucrose with ethanol (Zimmermann & Rutherford, 1986). The interconversion between the $g = 4$ and $g = 2$ multiline signals is influenced by choice of cryoprotectant; however, the effect of cryoprotectant on the magnetic properties of the Mn cluster is not fully understood.

Given the sensitivity of the interconversion between the $g = 4$ and $g = 2$ signals to cryoprotectant and temperature of illumination for generation of the S_2 state, the stabilization of the $g = 4$ signal relative to the $g = 2$ signal alone does not imply direct NH_3 ligation to Mn. In fact, Beck and Brudvig (1986) proposed that the lack of a significant alteration in the NH_3-stabilized $g = 4$ signal in nonoriented preparations in ethylene glycol indicates that NH_3 may effect the stabilization of the $g = 4$ signal by binding to a site in close proximity, but not directly on the Mn cluster. However, using PSII membranes prepared with sucrose as cryoprotectant, Andréasson et al. (1988) observed a narrowing of the $g = 4$ signal from a peak-to-peak width of 360 to 280 G and a shift of the baseline crossing point of the powder signal from $g = 4.1$ to $g = 4.2$ upon binding of NH_3. Nevertheless, these changes were small compared to, for example, the alteration in the $g = 2$ multiline signal induced by the binding of NH_3 at the non-Cl^- site.

Comparison of the oriented $g = 4$ spectra in Figure 1A,B shows that NH_3 binding at the Cl^- site significantly alters the $g = 4$ signal. This alteration remained masked in the broad featureless powder spectra observed in studies on nonoriented ammonia-treated samples. The improved resolution of the oriented spectra allows us to address the ways in which NH_3 binding alters the spectra. The binding of NH_3 may reduce the g anisotropy of the spectrum by inducing an alteration in the zero-field splitting parameters. Also, the binding of NH_3 may induce a significant change in the intracluster Mn exchange interactions, which could be reflected in a nonuniform reduction in the Mn hyperfine couplings. This would also induce a narrowing of the signal and could permit the resolution of hyperfine features. Both possible modes of alteration by binding of NH_3—an alteration in the zero-field splittings or a significant change in the Mn exchange couplings that could be reflected in reduced hyperfine couplings—are most directly explained by ligation of NH_3 to the Mn site. Most likely, a combination of these two effects results from the binding of NH_3. NH_3 coordination to the Mn complex

probably alters the symmetry of the complex, inducing a change in the zero-field splitting parameters, and at the same time alters the Mn exchange couplings. Such a change in Mn exchange couplings also accounts for the NH_3-induced stabilization of the $g = 4$ signal form of the Mn cluster relative to the $g = 2$ multiline form, because the interconversion between the forms most likely results from a modulation of the exchange couplings within the tetranuclear Mn complex.

We note that the inability to observe differences in the $g = 4$ spectra between $^{14}NH_4Cl$ preparations and $^{15}NH_4Cl$ preparations does not exclude the possibility that NH_3 coordinates directly to the Mn at this Cl^- site. In the case of NH_3 ligation to the non-Cl^- Mn site, the continuous-wave EPR spectra did not resolve any differences in the ammonia-altered $g = 2$ multiline spectra between preparations treated with $^{14}NH_4Cl$ or $^{15}NH_4Cl$ (Beck et al., 1986). Also, in the Mn_2-(III,IV) di-μ-oxo-bridged bipyridine compound, the magnitude of the isotropic hyperfine coupling of nitrogen ligands has been determined to be less than 1 G by ESEEM spectroscopy (Britt, 1988).

Studies of the Anisotropy in the g = 2 Multiline Signal. Previous studies of anisotropy in the $g = 2$ multiline signal obtained from oriented PSII membranes (prepared without ammonia treatment) at X band showed small shifts in the lowest field peaks of the spectrum, which were attributed to a small amount of g and/or hyperfine anisotropy (Rutherford, 1985). Studies on PSII membranes at S band (Haddy et al., 1989) and Q band (Hansson et al., 1987) indicated that hyperfine anisotropy was dominant in the $g = 2$ multiline signal, with little g anisotropy present.

The major difference between the data presented in Figure 3 and previous studies of the anisotropy in the $g = 2$ multiline signal is the improved resolution of the peaks in the wings of the spectrum. The orientation-dependent changes in the amplitudes of the lowest field peaks of the signal (maximal at 0°) and field positions of the highest field peaks of the signal indicate that considerable anisotropy exists in the wings of the signal. The anisotropy in the wings of the signal accounts for the absence of these extreme features of the signal in the powder spectrum obtained from a nonoriented sample (Figure 3). Because the effects of g anisotropy are expected to be most pronounced in the wings of the signal, the data presented here suggest that the g tensor for the $g = 2$ multiline signal may be more anisotropic than previously thought.

We have also been able to examine the orientation dependence of the high-field region ($g < 2$) of the spectrum because the annealing procedure in the presence of PPBQ eliminates the $g = 1.8$–1.9 $Fe^{2+}Q_A^-$ acceptor signal. Greater anisotropy exists in this region of the spectrum than in the low-field side of the signal. Powder Mn hyperfine peaks and shoulders can be partially decomposed into components that are better resolved at either 0° or 90°. The data of Figure 3 show that the origin of many small features superimposed on the major hyperfine peaks of the signal results primarily from anisotropic Mn hyperfine transitions and not superhyperfine couplings from Mn ligands. S band measurements (Haddy et al., 1989) showed that hyperfine anisotropy was present in the signal; the anisotropy observed in the peaks and shoulders of the spectra in Figure 3 probably has its origin in a combination of anisotropy in the hyperfine and g tensors.

The data in Figure 4 are, to our knowledge, the results of the first investigation of anisotropy in the NH_3-altered $g = 2$ multiline signal. This altered $g = 2$ multiline signal results from direct ligation of an NH_3-derived ligand to the Mn cluster, as postulated by Beck et al. (1986) and proved by Britt et al. (1989). The ESEEM data are suggestive that the ammonia coordinates to the complex as an NH_2 bridge between two Mn atoms or between Mn and Ca (Britt et al., 1989). In this study, we investigated the possible anisotropic effects on the NH_3-altered $g = 2$ multiline signal induced by ammonia binding to the Mn cluster. The oriented spectra of the NH_3-altered $g = 2$ multiline signal in Figure 4 can be interpreted in terms of a model in which anisotropy in the g tensor accounts for the anisotropic behavior of the peaks in the wings of the spectra. We assign the principal axes of the g tensor ($g_z > g_y > g_x$) to be such that g_y is oriented in the membrane plane, while g_x and g_z take on some intermediate orientation between 0° and 90° with respect to the membrane normal. From this assignment, we expect the greatest contribution to the 90° spectrum to be from the g_y component of the g tensor, with lesser contributions from the g_z and g_x components. This assignment is expected to be most noticeable in the wings of the 90° spectrum, where the g_z and g_x components contribute the most to the lowest and highest field peaks of the spectrum, respectively. Consistent with this model, Figure 4 shows a greater number of peaks in the wings of the 0° and 45° spectra than in the 90° spectrum, while the central peaks (near $g \approx 2$, where g_y is expected to make the greatest contribution to the spectrum) show significantly greater amplitude and resolution at 90°. The orientation dependence data on the NH_3-altered multiline signal are suggestive of some g anisotropy in the signal.

Conclusions

In this paper, we have discussed the $g = 4$ and $g = 2$ multiline EPR signals arising from the Mn site of the OEC in oriented preparations of PSII membranes. The observation of a $g = 4$ multiline signal from oriented ammonia-treated membranes strongly implicates a tetranuclear Mn cluster origin for the $g = 4$ signal. Simplified vector coupling calculations of the expected Mn hyperfine couplings in such a complex together with computer simulations of the spectrum are consistent with the assignment of the signal to an $S = 5/2$ or $S = 3/2$ ground state of a Mn tetramer. The orientation dependence data on the NH_3-modified $g = 4$ signal show that the g tensor for the signal is anisotropic and that the data are more consistent with an $S = 5/2$ assignment. The significant changes in the oriented $g = 4$ spectra induced by NH_3 binding to the Cl^- site of the OEC are suggestive of direct ligation of NH_3 to the Mn cluster. The orientation dependence data on the $g = 2$ multiline signal are indicative of some anisotropy in the g tensor.

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Cholesterol Heterogeneity in the Plasma Membrane of Epithelial Cells

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ABSTRACT: The distribution of cholesterol in the plasma membrane of epithelial cells has been determined using renal brush border vesicles as a model. In brush borders treated with *Brevibacterium sp.* or *Nocardia erythropolis* cholesterol oxidases, a significant fraction of the free cholesterol was oxidized rapidily, without glutaraldehyde fixation, and the remaining cholesterol was oxidized at a slower rate. The size of the readily accessible cholesterol pool, however, depended on the enzyme used, varying from 16% of the total in membranes treated with *N. erythropolis* oxidase, to 27% using the *Brevibacterium sp.* enzyme. The slowly accessible pool detected by the *Brevibacterium* oxidase was suppressed upon sphingomyelinase addition. On the other hand, the restricted activity of the *Nocardia* oxidase might depend on phosphatidylcholine/cholesterol interactions. These results indicate that cholesterol distribution is heterogeneous in intact renal brush border vesicles. They suggest that, as proposed for model systems [Demel, R. A., Jansen, J. W. C. M., van Dijck, P. W. M., & van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta 465*, 1–10], preferential interactions between some classes of phospholipids and cholesterol define cholesterol pools in the plasma membrane of epithelial cells.

Existence of cholesterol domains in the plasma membrane of epithelial cells is still under debate (Van Meer, 1987). So far, only in intestinal cells has a heterogeneous distribution of cholesterol been reported. This heterogeneity was proposed to result from lipid/protein interactions, with more than two-thirds of the cholesterol associated with a membrane protein fraction (Bloj & Zilversmit, 1982). In renal epithelial cells, modification of the cholesterol content of the apical membrane (brush borders) modulates transport activities (Molitoris et al., 1985; Levi et al., 1990). The distribution of cholesterol in this membrane, however, remains unknown.

As in intestinal cells, significant differences exist between the lipid order of the apical and basolateral domains of the

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