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S-Band EPR Studies of the S_2-State Multiline Signal from the Photosynthetic Oxygen-Evolving Complex†

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ABSTRACT: The S_2-state multiline EPR signal observed in photosynthetic membrane preparations has been previously well characterized at X-band frequencies (9.1-9.5 GHz). These studies have indicated that the signal, centered at $g = 2$, arises from a multinuclear mixed-valence Mn center of the O_2-evolving complex. In the present study, the multiline EPR signal from spinach photosystem II enriched membranes is characterized at an S-band frequency (3.9 GHz). At this lower frequency, the resolution and complexity of the signal increase markedly compared with its appearance in the X-band. While the multiline signal covers similar magnetic field ranges at the two frequencies, the S-band signal has a greater number of lines, narrower line widths, and a different overall appearance. Replacement of Cl^- with Br^- or ^1H_2O with ^2H_2O in the buffer shows that neither exchangeable Cl^- nor protons cause superhyperfine structure in the S-band multiline signal. Membrane preparations oriented on mylar sheets show dependence of the S-band signal on the angle between the mylar sheet normal and the magnetic field direction, indicating that the multiplicity of lines is in part due to signal anisotropy. The results, combined with previous work at X-band, indicate that a minimal working model for the species responsible for the multiline signal is a mixed-valence binuclear Mn complex with an anisotropic hyperfine interaction that includes second-order contributions.

The multiline EPR signal, centered at $g = 2$, is observed in photosynthetic membrane preparations in the S_2-state, one of several oxidative intermediates (S_0 through S_4) of the O_2-evolving system. Following its discovery (Dismukes & Siderer, 1981), another S_2-state signal at $g = 4.1$ was soon found (Casey & Sauer, 1984; Zimmermann & Rutherford, 1984). These two remain the only known EPR signals that have been positively identified with any of the S-states of the O_2-evolving complex. Due to the efforts undertaken in several laboratories, the multiline signal has been well characterized at X-band frequencies (Dismukes et al., 1982; Hansson et al., 1987; de Paula et al., 1987; Zimmermann & Rutherford, 1986). However, despite these extensive studies, our understanding of the origin of this signal, which is necessary for an understanding of the chemical mechanism of O_2 evolution, is not complete.

The X-band multiline signal covers a span of about 150 mT and consists of 16-20 main lines that are approximately evenly spaced and have numerous small peaks and shoulders. Models that explain the signal's appearance as arising from monomeric Mn with a small zero-field splitting have been ruled out on the basis of a lack of large-scale signal anisotropy (Hansson et al., 1984; Rutherford, 1985). Instead, the signal is thought to arise from a mixed-valence multinuclear Mn complex. Models for bi-, tri-, and tetranuclear Mn clusters have been proposed (Dismukes et al., 1982; Andréasson et al., 1983; Hansson et al., 1987; de Paula et al., 1987).

In order to understand the source of the many small peaks and shoulders of the multiline signal, investigations have been made into the influence of possible exchangeable ligands to Mn. These include the substrate water and the anion Cl^-, which is required for O_2 evolution (Critchley, 1985). Substitution of water ^{16}O with the magnetic ^{17}O nucleus produced broadening of details of the signal (Hansson et al., 1986; Andréasson et al., 1988). This result implies that oxygen from water becomes closely associated with the Mn center by the time it is in the S_2-state. Similar investigations using deuterated water have been less clear. While no effect of ^2H_2O was found by one laboratory (Yachandra et al., 1986), an increase in the resolution of the multiline signal's small peaks and shoulders was found by another (Nugent, 1987). In the case of Cl^-, substitution experiments using Br^- as the substituting ion revealed no change in the multiline signal from spinach photosystem II (PSII)[1] preparations (Yachandra et al., 1986). Thus, although H_2O may have subtle effects on the line width of the multiline signal, neither H_2O nor exchangeable Cl^- has been found to be responsible for the small

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[1] Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; OEC, oxygen-evolving complex; PPBQ, phenyl-*p*-benzoquinone; PSII, photosystem II.

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peaks and shoulders of the signal.

Orientation dependence of the X-band multiline signal has been studied by using chloroplasts frozen in a magnetic field (Hansson et al., 1984) and PSII membranes prepared on mylar sheets (Rutherford, 1985), procedures which align membrane fragments. The latter study revealed a dependence on the angle between the membrane normal and the magnetic field direction, observed as small changes in the positions of some of the low-field peaks. The degree of orientation dependence was too small to account for major features of the X-band signal, but the results indicated that at least some multiline peaks include anisotropic components.

While most characterizations of the multiline signal have been done at X-band frequencies (9 GHz), much more information about the signal's origin can be gained by combining X-band spectra with those taken at other frequencies. At higher frequencies, hyperfine splitting, which is constant, can be differentiated from g components of a powder pattern. Thus, a study including three X-band frequencies and a Q-band frequency (34 GHz) revealed that the multiline signal is centered at $g = 1.982 \pm 0.002$ and that it does not involve significant g anisotropy (Hansson et al., 1987). By working at lower frequencies, on the other hand, hyperfine components of a signal may be better resolved since g-strain broadening, which is proportional to the microwave frequency, is decreased. In addition, second-order hyperfine coupling, which is inversely proportional to the frequency, increases, making a more significant contribution to hyperfine splitting.

In the work presented here, the S_2-state multiline signal from PSII-enriched membranes has been characterized at the S-band frequency of 3.9 GHz. The signal has been found to be more complex than its X-band counterpart with a greater number of lines and narrower line widths. Because changes in the multiline signal may be more easily observed in these better resolved spectra, investigations into the orientation dependence of the signal and the effects of exchangeable Cl^- ions and water protons have been undertaken. The present study indicates that part of the signal's complexity can be attributed to hyperfine anisotropy, while neither Cl^- nor water protons have a significant influence on its overall structure.

Materials and Methods

PSII-Enriched Membrane Preparations. Photosystem II enriched thylakoid membranes were prepared from house-grown spinach by the Triton X-100 extraction method as described previously [Franzén et al. (1985) modified from Berthold et al. (1981)]. Preparations were resuspended in 20 mM Mes–NaOH, pH 6.3, 15 mM NaCl, 5 mM $MgCl_2$, and 400 mM sucrose at a concentration of about 10 mg of Chl/mL and stored at 77 K in liquid N_2. O_2-Evolving activity was typically 600 ± 100 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} measured at 25 °C with a Clark-type O_2 electrode using the acceptor PPBQ.

Preparation of ^2H_2O-Substituted PSII Membranes. PSII-enriched membranes were washed once with buffer containing 20 mM Mes, pH 6.3, 15 mM NaCl, 5 mM $MgCl_2$, and 400 mM sucrose prepared with either distilled ^1H_2O or ^2H_2O (CIBA-GEIGY, >99% isotopic purity). The membranes were diluted to 0.4 mg of Chl/mL with the same buffers and incubated at 6 °C for about 5 h in the presence of 25 μM PPBQ. (The ^2H_2O buffer freezes if left on ice.) During this time, the two samples were illuminated for 6 min each (in periods of 30 s to prevent warming) while swirling; illumination was with white light from a 250-W tungsten halogen lamp, passed through 8 cm of water and an infrared-reflecting filter for a resulting intensity of about 2000 W/m^2. The membranes were then concentrated by centrifugation and transferred to EPR tubes.

In a second method, PSII membranes were diluted to about 0.8–1 mg of Chl/mL with either the ^2H_2O or the ^1H_2O buffer given above and incubated at 6 °C for about 2 h. After being washed and rediluted, the membranes were incubated again at 6 °C for 16 h under room light, then centrifuged, and transferred to EPR tubes.

For both of these methods, the O_2-evolving activities of the ^2H_2O- and ^1H_2O-treated membranes, measured in the ^1H_2O buffer system, were within the experimental error of the untreated preparations.

Preparation of Br^--Substituted PSII Membranes. PSII-enriched membranes were thawed from storage and washed twice with buffer containing 20 mM Mes, pH 6.3, 400 mM sucrose, and either 15 mM NaBr and 5 mM $MgBr_2$ or 15 mM NaCl and 5 mM $MgCl_2$. The membranes were diluted to about 0.5 mg of Chl/mL with the same buffers and incubated on ice for about 4 h in the presence of 35 μM PPBQ. During this time, the two samples were illuminated for 12 min each with the halogen lamp as described above for the ^2H_2O- and ^1H_2O-treated samples. The membranes were washed once more with the appropriate buffers before being transferred to EPR tubes. The O_2-evolving activities of the Br^-- and Cl^--treated membranes, measured in their own buffer systems, were within the experimental error of the untreated preparation.

Preparation of Oriented PSII Membranes. Orientation of PSII membrane preparations was achieved by slow drying onto mylar using a method similar to those described previously (Prince et al., 1980; Rutherford, 1985). A 12 × 10 cm^2 mylar sheet was washed with detergent and water, wiped with 95% ethanol, and dried. PSII membranes were thawed from storage and, after dark-adapting on ice for 45 min, were thickly painted onto the mylar sheet. The preparation was dried at room temperature in the dark for about 18 h in a container through which nitrogen gas flowed. The mylar sheet was cut into strips of about 3.5 × 0.7 cm^2, and 25–30 of these, holding about 0.9 g of partially dehydrated PSII membranes, were layered inside an S-band tube under dim green light. The sample was stored in liquid nitrogen until use.

PSII Core Preparations. PSII membrane preparations were purified further essentially as described for the "reaction center complex" by Ghanotakis and co-workers (Ghanotakis et al., 1987), with modification as follows. The two dialysis steps were replaced by precipitation with poly(ethylene glycol) 1500 (N. Bowlby, personal communication), followed by an extra wash. The preparation was resuspended in 40 mM Pipes–NaOH, pH 6.8, 15 mM NaCl, 10 mM $CaCl_2$, and 400 mM sucrose; this higher pH was found to enhance the activity by about 10%. O_2-Evolving activity was 2230 ± 60 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} using the acceptor PPBQ. The preparation, as judged by SDS–PAGE (Chua, 1980), had much the same appearance as the preparation described by Ghanotakis and co-workers (Ghanotakis et al., 1987), with few light-harvesting complex proteins in the 25–30-kDa range and no visible 23- and 17-kDa proteins.

EPR Sample Illumination. Illumination of EPR samples was carried out either at 200 K, in an ethanol–dry ice bath, or while freezing, as indicated. Illumination was with white light from a 250-W tungsten lamp, passed through 10 cm of water and an infrared-reflecting filter for a resulting intensity of about 2000 W/m^2.

EPR Measurements. EPR spectroscopy was performed with a Bruker ER 200D-SRC spectrometer interfaced to an

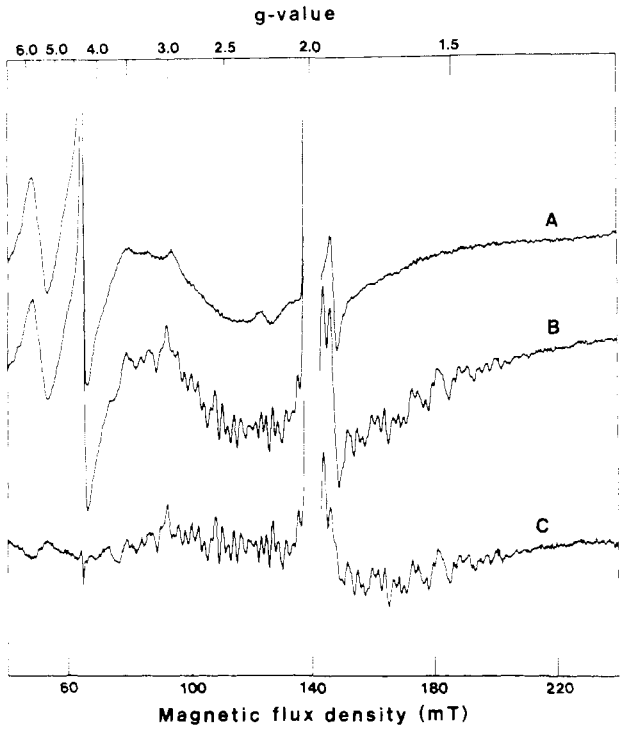

FIGURE 1: S-Band (3.91 GHz) EPR spectra showing the light induction of the S_2-state multiline signal of PSII-enriched membranes. Spectra shown are of (A) dark-adapted sample, (B) illuminated sample, and (C) difference between spectra B and A. After dark-adaptation of the 8.8 mg of Chl/mL sample, 2% ethanol and 100 μM DCMU were added before freezing to liquid N_2 temperature. Illumination was carried out at 200 K for 30 min. EPR conditions, other than those stated under Materials and Methods, included a microwave power of 13 mW, a modulation amplitude of about 0.4 mT, and a temperature of 10 K.

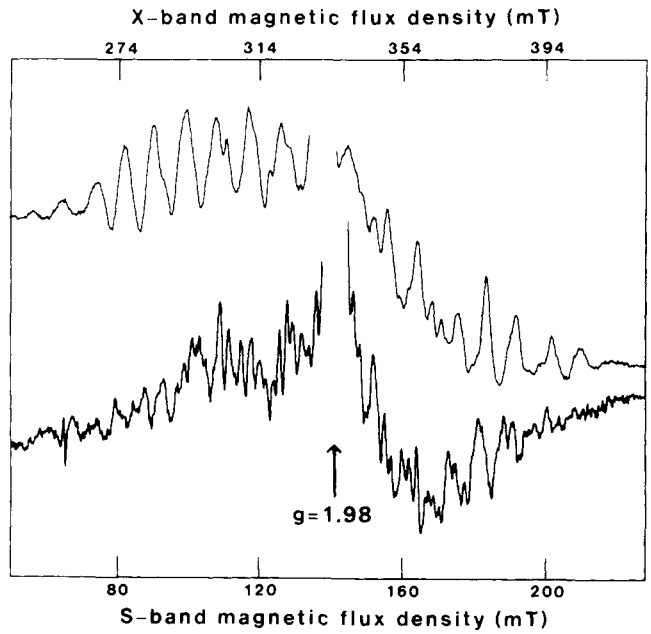

FIGURE 2: Comparison of X-band (top) and S-band (bottom) multiline signals of a PSII core preparation. The spectra show 200 mT scan ranges and coincide at $g = 1.98$. The 5.6 mg of Chl/mL samples were dark-adapted and 2% ethanol and 100 μM DCMU (in DMSO solvent) added before freezing. The S-band sample was illuminated for 1 h and the X-band sample for 4 min at 200 K. S-Band EPR conditions included a microwave power of 100 mW, a modulation amplitude of about 0.4 mT, and a temperature of 15 K; X-band EPR conditions included a microwave power of 20 mW, a modulation amplitude of 1.0 mT, and a temperature of 12 K. Both spectra represent the difference between spectra taken after and before illumination.

Aspect 2000 minicomputer and equipped with an Oxford Instruments ESR-9 helium flow cryostat.

All S-band measurements employed a Bruker ER 061 SR microwave bridge and an ER 6102 SR reentrant cavity and were taken by using a microwave frequency of 3.91 GHz and a modulation frequency of 12.5 kHz. To accommodate a larger sample size, the ESR-9 cryostat was modified with a homemade quartz insert. A typical quartz S-band tube had an inner diameter of about 8 mm and held about 1.5 mL of sample.

X-band measurements were carried out using a microwave frequency of 9.38 GHz and a modulation frequency of 100 kHz.

RESULTS

S-Band Multiline Spectrum. The S_2-state multiline signal at 3.9 GHz was found to consist of 40–50 lines extending from about 70 to 210 mT (Figure 1); thus, while the overall width of the signal on a field scale remained about the same as at X-band, the number of lines increased (Figure 2). Peak-to-peak spacings at S-band were commonly about 2–2.5 mT and in some cases as little as 1.5 mT. The lines appeared to be fairly evenly spaced except at the outermost regions of the spectra where groupings of three or more lines were observed. No clear correlations could be found between S-band and X-band peaks and shoulders. In general, S-band spectra showed greater complexity and resolution than their X-band counterparts.

Numerous lines from other components of the PSII-enriched membranes were visible in the S-band spectra taken before and after sample illumination (see Figure 1). This is because at the lower frequency lines due to different g values become compressed on a field scale whereas hyperfine splittings, such as that from the multiline signal, remain about the same. Lines not associated with the multiline signal were identified on the basis of their reported X-band g values and orientation dependences (Rutherford, 1985; Aasa et al., 1981). Spectra of both dark-adapted and illuminated samples contained an intense line at $g = 2.0$ (most of which was deleted for the figures) due to signal II slow, a strong line at $g = 4.3$ from nonspecifically associated rhombic Fe(III), and a weaker line at $g = 1.90$ attributed to the Rieske FeS center from a trace of contaminating cytochrome b_6f complex. A signal around $g = 5$–6, probably from high-spin Fe, was also visible. Spectra of the nonilluminated samples also typically showed the g_z and g_y components of low-potential cytochrome b_{559} at $g = 2.97$ and $g = 2.22$, respectively. After illumination, lines characteristic of high-potential cytochrome b_{559} were sometimes visible at $g = 3.05$ and $g = 2.18$. A light-induced line at $g = 1.93$ is believed to have arisen from the FeS center A of PSI; comparison of this signal with the center A signal of thylakoid membrane preparations indicated that about 3% of the chlorophyll in the PSII membrane preparation represented PSI contamination. A light-induced signal at $g = 1.90$ was usually observed; its origin is unknown, but a similar signal is believed to be due to FeQ_A^- (Rutherford & Zimmermann, 1984), although this component is usually favored by a higher pH than was used here. Two other light-induced signals from FeQ_A^- were sometimes observed at $g = 1.82$ and $g = 1.66$.

Illumination of the S-band samples was found to be problematic because of the large inner diameter of the tubes (8 mm). About 2% ethanol was routinely added to the PSII-enriched membranes, which were at about 10 mg of Chl/mL, in order to maximize the intensity of the multiline signal at the expense of the $g = 4.1$ signal (Zimmermann & Rutherford,

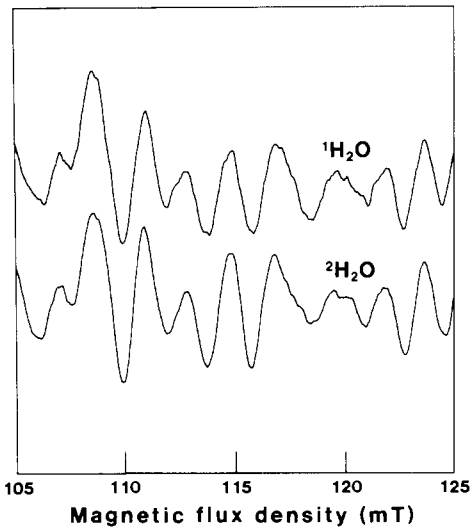

FIGURE 3: S-Band multiline signal (low-field portion) of ^2H_2O-substituted (bottom) and ^1H_2O control (top) PSII membranes. The 10–11 mg of Chl/mL samples were prepared with ^2H_2O or ^1H_2O solvent in the presence of an acceptor as described under Materials and Methods. Ethanol and DCMU additions, freezing, illumination, and EPR conditions were as given for the S-band sample in the legend to Figure 2. The amplitude of the ^1H_2O control sample, which had a smaller volume, was increased to correspond to that of the ^2H_2O sample.

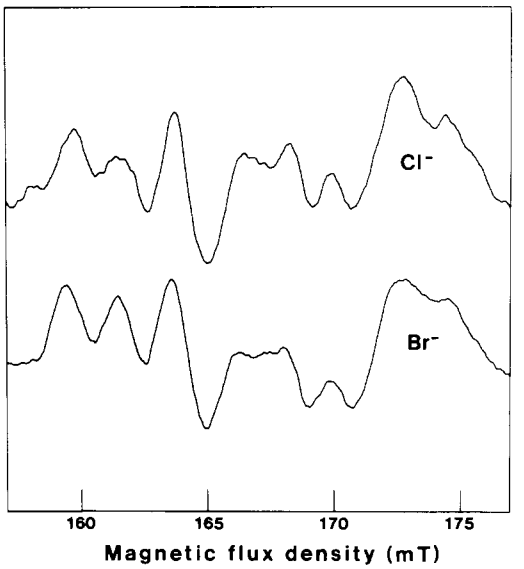

FIGURE 4: S-Band multiline signal (high-field portion) of Br^--substituted (bottom) and Cl^- control (top) PSII membranes. The 11.4 and 12.8 mg of Chl/mL samples were prepared with Br^- or Cl^- buffers, respectively, in the presence of an acceptor as described under Materials and Methods. Ethanol and DCMU additions, freezing, illumination, and EPR conditions were as given in the legend to Figure 1, except that a microwave power of 20 mW was used.

1986). (Thus, the $g = 4.1$ signal was not observed in these experiments, although in the absence of ethanol a broad signal centered at about $g = 4.1$ was observed.) However, samples to which only ethanol had been added required more than 1 h of illumination at 200 K to develop the multiline signal to its fullest. It was believed that although this illumination time was needed for enough light to reach the center of the sample, overillumination, resulting in advancement to the S_3-state (de Paula et al., 1985), occurred at the outer regions. With the addition of about 100 μM DCMU, S-band samples required no more than 30 min of illumination for maximal development of the multiline signal.

A comparison was made between the S-band multiline signals of PSII-enriched membranes (Figure 1) and a PSII core preparation (Figure 2). Since the PSII core preparation contained fewer chlorophylls per reaction center (70–75) than PSII-enriched membranes (about 200) (Bowlby et al., 1988), it was thought that achievement of the S_2-state by illumination at 200 K might be less hindered by the thickness of the S-band samples using the core preparation. The S-band multiline spectra of the two preparations were found to be essentially the same, with lines showing similar positions and relative intensities. The few differences observed were largely in the resolution of lines and may therefore be related to different responses to the added ethanol. A significant increase in signal size was not observed using the core preparation, indicating that illumination efficiency was not improved.

Effects of ^2H_2O and Br^- Substitution. Substitution of exchangeable water protons and Cl^- was done in order to ascertain whether any of the numerous S-band multiline peaks represented splittings due to these nuclei. Replacement of protons with deuterons is expected to decrease the separation between superhyperfine lines by a factor of 0.153 while increasing the number of superhyperfine lines from two to three per proton ligand [since $I(^1H) = {}^1/_2$ and $I(^2H) = 1$]. As a result, a peak made up of unresolved proton superhyperfine lines may appear narrower or more resolved if ^2H replaces ^1H. However, in the experiments undertaken here, no notable differences in peak positions or line widths were found when exchangeable ^2H_2O was substituted for ^1H_2O in PSII membrane preparations (Figure 3). For the first replacement method, in which the ^2H_2O-washed and ^1H_2O control membranes were illuminated in the presence of an acceptor, the S_2-state was produced in the dark-adapted samples by illumination during freezing as well as at 200 K (shown in Figure 3). Duplicate samples examined at X-band frequency after producing the S_2-state by both illumination methods also showed almost no difference in the signal. In the second replacement method, in which the membranes were incubated in ^2H_2O or control buffer overnight, the S_2-state was produced by illumination at 200 K. In all cases, variations in signal amplitude between $^1H_2O/^2H_2O$ pairs could be attributed to differences in sample size or concentration.

Replacement of Cl^- with Br^- is expected to increase superhyperfine splitting due to the halide by as much as a factor of 3, if coupling to the halide ligand is not changed, while the number of lines remains the same (since the isotopes ^{35}Cl, ^{37}Cl, ^{79}Br, and ^{81}Br all have $I = {}^3/_2$); a comparison of superhyperfine splittings due to Cl and Br in several transition-metal complexes is given by Yachandra and co-workers (Yachandra et al., 1986). A peak made up of unresolved Cl superhyperfine lines may appear broadened if Br substitutes for Cl. However, little difference was found between the S-band multiline spectra of Br^--substituted and Cl^- control PSII membranes (Figure 4). In one experiment, a few lines on the low-field side of the signal appeared to have shifted as a result of the Br^- treatment, but this effect was found to be related to the addition of ethanol. When a higher concentration of ethanol was used (4%), the two PSII membrane samples showed similar S-band multiline spectra.

Orientation Studies. Oriented PSII membrane samples were prepared on mylar sheets in order to investigate whether the complexity of the S-band multiline signal was in part due to signal anisotropy. The success of oriented sample preparation was assessed through the low-potential cytochrome b_{559} signals taken before sample illumination: when the mylar sheet normal was at an angle of 0° to the magnetic field direction, the g_y component of the signal at $g = 2.22$ was maximized

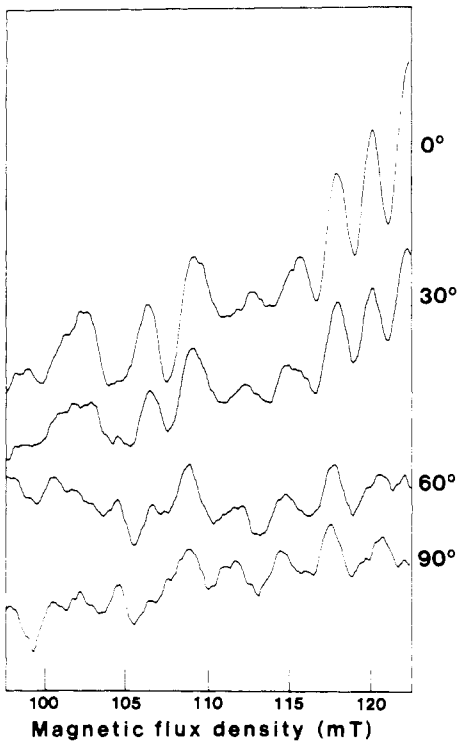

FIGURE 5: S-Band multiline signal (low-field portion) of oriented PSII membranes at angles of 0°, 30°, 60°, and 90° (top to bottom) between the membrane normal and the magnetic field. The PSII membrane sample was prepared on mylar as described under Materials and Methods and dark-adapted before freezing in liquid N_2. Illumination was carried out at 200 K for 30 min. The sloping base lines of the top spectra are due to the g_y component of cytochrome b_{559}, a portion of which underlies this region of the multiline spectrum. EPR conditions included a microwave power of 25 mW, a modulation amplitude of about 0.4 mT, and a temperature of 11 K.

while the g_z component at $g = 2.97$ was absent, whereas at an angle of 90°, the g_z component was maximized while the g_y component was absent. This orientation dependence matched that reported previously for oriented PSII membranes studied at X-band frequency (Rutherford, 1985). The cytochrome b_{559} signals were used to confirm and refine the reference angle setting (0° in this case).

The S-band multiline signal of PSII membranes showed a marked degree of orientation dependence (Figure 5). Most peaks changed in amplitude as the angle with the magnetic field varied, with many completely disappearing at either 0° or 90°. Apparent peak shifts could be accounted for by the simultaneous decrease and increase in the amplitudes of at least two overlapping peaks, since in these cases broadened or shouldered peaks were observed at intermediate angles. At either 0° or 90°, the number of lines observed was about three-fourths that of the nonoriented spectra.

Discussion

At S-band frequency (3.9 GHz) the S_2-state multiline signal has a greater complexity and is better resolved than at X-band (9 GHz). Although the magnetic field range covered by the signal (about 150 mT) is similar at the two frequencies, S-band spectra show 40–50 separate lines with spacings of about 2–2.5 mT, whereas X-band spectra show 16–20 main lines with spacings of 7–9 mT. This increase in the number of lines is the most striking feature of the S-band spectra.

While a model for the Mn cluster involving two antiferromagnetically coupled ions with different isotropic hyperfine couplings can account for the number of lines observed at X-band, it cannot in itself explain the observation of more than 36 lines at S-band. The model must be extended to include other factors. One possibility is that the Mn cluster includes more than 2 Mn atoms; for example, a cluster of 3 Mn ions could give rise to up to 216 lines. A second possibility is superhyperfine splitting from liganding magnetic nuclei; for example, coupling to a Cl nucleus could increase the number of lines by a factor of $2I + 1 = 4$. Finally, g value or hyperfine anisotropy could increase the number of lines by a factor of 2, for axial symmetry, or 3, for rhombic symmetry. The S-band investigations presented here had the goal of testing some of these possibilities.

The lack of a discernible difference between the spectra of ^2H_2O-washed and ^1H_2O control PSII membranes shows that none of the lines of the S-band multiline signal are caused by superhyperfine splitting by exchangeable hydrogen nuclei from water molecules involved in O_2 production. To facilitate replacement of ^1H_2O with ^2H_2O in the O_2-evolving system, the PSII membranes were in one case illuminated in the presence of an acceptor, to ensure numerous system turnovers before dark-adaptation, and in the other case incubated overnight, a method more similar to that employed by Nugent (1987). The absence of an effect on the S-band multiline signal in both cases is in support of the work of Yachandra and co-workers (Yachandra et al., 1986), who found no change in the X-band multiline signal of PSII membranes when ^2H_2O replaced ^1H_2O. On the other hand, Nugent (1987) observed an increase in the resolution of the smaller peaks and shoulders of the X-band signal. This effect was most apparent when the membranes were illuminated during freezing, suggesting that the protons of water are not associated with the Mn cluster in dark-adapted samples but become associated with it during the S_2-state or the transition from the S_1- to the S_2-state. However, since the broadening effects of oxygen of $H_2{}^{17}O$ were observed in samples illuminated at 200 K (Hansson et al., 1986), effects due to water protons are expected to be observable under these conditions as well. In the work presented here, no significant difference was found between membranes illuminated during freezing or at 200 K. Thus, the major difference between the treatments employed here and in Nugent's work is that in the latter case the membranes were washed at the elevated pH of 7.5 and kept at that pH for many of the EPR studies. This suggests that the effect of deuteron replacement of protons observed by Nugent arose from water or hydroxyl groups which function in a capacity other than O_2 evolution.

The lack of a notable effect resulting from the replacement of Cl^- with Br^- indicates that none of the lines of the S-band multiline signal are due to the presence of exchangeable Cl^- within the coordination sphere of Mn. This conclusion supports that the Yachandra and co-workers (Yachandra et al., 1986), who studied the effects of halides on the X-band multiline signal of PSII-enriched membranes prepared from Cl^--depleted chloroplasts. In the present S-band study, Cl^- was exchanged for Br^- by illuminating in the presence of an acceptor before dark-adaptation. These results do not rule out the possibility that Cl^- may bind to the Mn cluster, since the coupling may be too small to be observed by this method, particularly if there is low covalency in the metal–ligand bonds.

Studies of the orientation dependence revealed marked anisotropy in the S-band multiline signal of PSII membranes oriented on mylar. This confirms the observation of Rutherford (1985), who reported small shifts in low-field X-band multiline peaks of similarly prepared samples. The effects of anisotropy at X-band are so slight as to be difficult to observe in chloroplasts oriented by freezing in a magnetic field (Hansson et al., 1984). At S-band frequency, however, the

anisotropy of the multiline signal is evident in changes in peak amplitude as the angle with the magnetic field varies. Oriented samples show fewer lines than nonoriented samples, indicating that the multitude of lines is in part due to signal anisotropy. This demonstration of orientation dependence shows that a model in which all g values and hyperfine constants are isotropic cannot adequately account for the multiline signal. The observation of significant signal anisotropy at S-band but not at X-band indicates that the degree of anisotropy must be greater than the observed line width (0.5–1 mT) but less than the distance between peaks (2–2.5 mT). The source of anisotropy is probably in the hyperfine coupling to at least one of the Mn nuclei; Mn(III) is the most likely ionic form to give rise to anisotropy because of the relatively high geometric inequivalence of occupied orbitals of the d^4 electronic configuration.

Finally, the increase in resolution and complexity of the S-band multiline signal compared to the X-band signal must be accounted for. Part of the increased resolution at S-band is probably due to a decrease in g-strain broadening, which is proportional to frequency. However, most of the numerous S-band lines are evenly spaced, and the correlation between S-band lines and X-band peaks and shoulders is not obvious. This indicates that part of the complexity of the S-band signal results from variable shifting of peaks as second-order hyperfine effects make more significant contributions. These effects will be more important at the lower frequency because second-order hyperfine splitting is inversely proportional to frequency. For example, upon going from X-band to S-band frequency, two antiferromagnetically coupled Mn nuclei with $S = {}^1/_2$ and different isotropic hyperfine constants of $A_1 =$ 18 mT and $A_2 = 8$ mT (Hansson & Andréasson, 1982) will show line shifts to lower field of between 1 and 9 mT due to second-order hyperfine effects alone. Lines that are next to one another may show differences in their shifts that are as much as 4–5 mT.

In summary, S-band spectra of the S_2-state multiline signal provide a new tool for the refinement of current models for the Mn cluster of the O_2-evolving complex. The higher resolution and greater complexity of the signal at S-band frequency increase the likelihood that factors influencing its structure can be detected. In the present study, no evidence has been found that exchangeable Cl^- or water protons cause superhyperfine structure. On the other hand, marked signal anisotropy has been observed at S-band. These data, taken with the results of earlier studies, suggest a minimal model for the species giving rise to the multiline signal. This would involve a mixed-valence binuclear Mn cluster with $S = {}^1/_2$; recent models have favored antiferromagnetically coupled Mn(III)–Mn(IV), because of results from X-ray absorption edge spectroscopy (Goodin et al., 1984) and absorbance difference spectroscopy measurements (Dekker et al., 1984a,b). No superhyperfine splitting due to exchangeable Cl^-, exchangeable protons, or liganding nitrogen atoms (Andréasson, 1989) needs to be accounted for. However, the model must include a hyperfine interaction with second-order contributions. In addition, the hyperfine interaction must account for signal anisotropy, which is expected to have its origin largely in the Mn(III) ion.

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