Multifrequency electron spin-echo envelope modulation studies of nitrogen ligation to the manganese cluster of photosystem II

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The CalEPR Center at UC-Davis (<http://brittepr.ucdavis.edu>) is equipped with five research grade electron paramagnetic resonance (EPR) instruments operating at various excitation frequencies between 8 and 130 GHz. Of particular note for this RSC meeting are two pulsed EPR spectrometers working at the intermediate microwave frequencies of 31 and 35 GHz. Previous lower frequency electron spin-echo envelope modulation (ESEEM) studies indicated that histidine nitrogen is electronically coupled to the Mn cluster in the S₂ state of photosystem II (PSII). However, the amplitude and resolution of the spectra were relatively poor at these low frequencies, precluding any in-depth analysis of the electronic structure properties of this closely associated nitrogen nucleus. With the intermediate frequency instruments, we are much closer to the ‘exact cancellation’ limit, which optimizes ESEEM spectra for hyperfine-coupled nuclei such as ¹⁴N and ¹⁵N. Herein, we report the results from ESEEM studies of both ¹⁴N- and ¹⁵N-labelled PSII at these two frequencies. Spectral simulations were constrained by both isotope datasets at both frequencies, with a focus on high-resolution spectral examination of the histidine ligation to the Mn cluster in the S₂ state.

Keywords: multifrequency electron spin-echo envelope modulation; histidine ligation; manganese cluster; photosystem II

1. INTRODUCTION

Photosystem II (PSII) is a multisubunit membrane-spanning protein that uses light energy to catalyse the production of molecular oxygen from water. This biochemical process takes place at the oxygen-evolving complex (OEC), which consists of a tetranuclear manganese cluster, a redox-active tyrosine (Y_Z) and two essential cofactors Ca²⁺ and Cl[−] (Debus 1992; McEvoy & Brudvig 2006). During catalytic turnover, a photooxidized P680⁺ chlorophyll molecule is reduced by Y_Z, which in turn is reduced by the Mn cluster. This chain of redox events is described by the S-state or Kok cycle (Kok *et al.* 1970) in which there are five S-state intermediates, with S₀ representing the most reduced state and S₄ the most oxidized state.

Throughout the past three decades, numerous biochemical and spectroscopic methods have been employed to probe the dynamic and complex nature of the OEC at all stages of the Kok cycle (Debus *et al.* 2005; Haumann *et al.* 2005; Kulik *et al.* 2005). Among the most important of these techniques is pulsed electron paramagnetic resonance (EPR). Two sub-categories of pulsed EPR, electron spin-echo envelope modulation (ESEEM) and electron spin-echo-electron

nuclear double resonance (ESE-ENDOR), have played significant roles in aiding the characterization of the OEC architecture, electronic structure, amino acid ligation and substrate/inhibitor binding (Peloquin *et al.* 2000; Britt *et al.* 2004).

While much information has been obtained from previous pulsed EPR experiments, most of the studies were done at relatively low X-band frequencies (8–12 GHz), thus offering limited resolution of any stronger couplings that may be present. However, the ability to apply ESE techniques over a broad band of microwave frequencies provides enhanced resolution of nuclear interactions within the vicinity of the Mn cluster of the OEC. In this paper, we report a mid-frequency (31–35 GHz) ESEEM study of imidazole nitrogen ligation to the Mn cluster poised in the S₂ state.

2. ESEEM SPECTROSCOPY

Pulsed EPR spectroscopy can be applied to overcome the dense superposition of EPR lines in complicated disordered paramagnetic systems. In the limit of unresolved hyperfine couplings, the number of EPR lines increases multiplicatively with the number of coupled nuclei, thus leading to broad featureless Gaussian line shapes. Such an example of this inhomogeneous line broadening is the EPR spectra of the S₂ state of PSII. While seemingly rich in structure, only 18–21 peaks of the possible 1296 EPR transitions from four

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^{55}Mn ($I=5/2$) couplings are resolved (Dismukes & Siderer 1981). These dominant Mn hyperfine couplings clearly suppress any weaker couplings one might expect from ligand nuclei such as ^{14}N , ^{15}N , ^1H and ^2H . The pulsed EPR method of ESEEM is sensitive to the weaker ligand couplings buried in the inhomogeneous broadened multiline signal.

ESEEM spectroscopy involves the application of two or more resonant microwave pulses to an unpaired electron spin system immersed in a static magnetic field. The ESEEM effect is a result of the microwave pulses inducing fully allowed electron spin transitions as well as 'semi-allowed' transitions of nuclear spins magnetically coupled to the electron spins. This induction of multiple spin transitions results in quantum mechanical coherences between the nuclear spin sublevels associated with the electron spin levels. These coherences manifest themselves as modulations of the electron spin-echo intensity as a function of the interpulse timing. Fourier transformation of the modulation pattern in the time domain of the echo envelope reveals the frequencies of the nuclear spin transitions. Analysis of the amplitude and frequency of these Fourier peaks can be used to determine the hyperfine and electric quadrupolar interactions of nuclei coupled to the unpaired electron spin.

The extent of modulation of the spin-echo, or the modulation depth, is limited by the inherent spin physics of the system under study and the detection instrumentation itself. As indicated above, the ESEEM effect is dependent upon the microwave pulse induction of both forbidden and semi-forbidden transitions. By dissection of the above argument, our first limitation is naturally instrumental, in that the bandwidth of the microwave pulse must span the superhyperfine manifold. Second, the semi-forbidden transition probability is directly proportional to the magnitude of the off-diagonal spin Hamiltonian terms in the electron and nuclear spin quantum number basis. Thus, for nuclei with a nuclear spin (I) = 1/2, anisotropic hyperfine couplings induce modulations in the time-domain ESEEM data. For nuclei with $I > 1/2$, the nuclear quadrupolar interaction further contributes to the off-diagonal elements of the spin Hamiltonian by mixing the m_I states. Finally, optimal modulation depth can be achieved by tuning the external magnetic field such that the nuclear Zeeman interaction cancels the hyperfine interaction in one electron spin manifold. In this 'exact cancellation' limit, the transition probabilities of the fully allowed and semi-allowed transitions are similar, and thus modulation depth is maximized. For $I=1$ nuclei, the cancellation condition gives rise to deep modulation patterns associated with pure quadrupolar transitions, as well as modulations from the other ^{14}N energy levels in which the nuclear Zeeman and hyperfine interactions sum together. The peak positions and line shapes in the frequency domain allow for full determination of the nuclear quadrupole and hyperfine interactions (Flanagan & Singel 1987).

To this end, two-pulse Hahn echo and three-pulse stimulated echo ESEEM experiments are employed on a high-power K_a -band (approx. 31 GHz) home-built pulsed EPR spectrometer and a Bruker pulsed Q-band

(approx. 34 GHz) spectrometer in order to study imidazole nitrogen ligation to the Mn cluster of the OEC.

(a) Multifrequency EPR

Over the past decade or so, multifrequency EPR has been applied to study numerous biological and synthetic systems. The push to higher magnetic fields and frequencies has provided significant g-anisotropy resolution (Matsuoka *et al.* 2006), ENDOR frequency resolution (Goldfarb 2006) and the accessibility to transition metal complexes with dominant zero-field splitting interactions (Krzystek *et al.* 2006). This is clearly evident by the inspection of the spin Hamiltonian described in the following equation:

$$\hat{\mathcal{H}} = \beta_e \hat{\mathbf{B}} \cdot \mathbf{g} \cdot \hat{\mathbf{S}} - g_n \beta_n \hat{\mathbf{I}} \cdot \hat{\mathbf{B}} + \hat{\mathbf{S}} \cdot \mathbf{A} \cdot \hat{\mathbf{I}} + \hat{\mathbf{I}} \cdot \mathbf{P} \cdot \hat{\mathbf{I}} + \hat{\mathbf{S}} \cdot \mathbf{D} \cdot \hat{\mathbf{S}}. \quad (2.1)$$

Here, the first two terms represent the magnetic field-dependent electronic and nuclear Zeeman interactions, respectively. The remaining three terms are as follows: the hyperfine; nuclear quadrupolar; and electronic zero-field splitting interactions.

However, a consequence of high-field EPR can be decreased sensitivity to the hyperfine (in some cases) and nuclear quadrupolar couplings (in most cases). Again, with reference to equation (2.1), as the field-dependent terms in the spin Hamiltonian begin to dominate, the non-field-dependent terms become less significant and essentially become only small perturbations to the spin system's Zeeman energy levels. While relatively small in magnitude, compared with other spin Hamiltonian terms, the hyperfine and quadrupolar couplings contain valuable information with respect to the ligand environment of transition metal complexes.

From the discussion above, clearly, there exists a delicate balance between the operational spectrometer frequency and experiments needed to probe the system under study. The beauty of this situation, however, is that access to multifrequency instrumentation allows the experimentalist to selectively enhance or suppress specific spin Hamiltonian interactions as needed. At the CalEPR Center at UC-Davis, we are well equipped with spectrometers that cover 9 (CW), 9–18 (pulsed), 31 (pulsed), 34 (pulsed) and 130 (pulsed) GHz. This broadband approach to EPR spectroscopy has shown great promise in furthering the understanding of the structure and function of paramagnetic biological systems.

(b) Multifrequency ESEEM

Nitrogen couplings to transition metals in biological and synthetic systems are key targets of ESEEM studies. This is due in part to the plethora of nitrogen containing amino acid side-chain residues that can potentially ligate metal centres (e.g. histidine, arginine, asparagine, lysine, glutamine). There are key metal cofactors, such as the haem group, which contain four ligand nitrogen atoms. As well, nitrogen-rich ligands are employed in many synthetic model complexes of bioinorganic systems (e.g. bipy, cyclam, bpea, phen). Furthermore, the quadrupole moment of the ^{14}N ($I=1$) nucleus tends to be large,

leading to subsequent m_I state mixing and, therefore, deep modulation in the time-domain ESEEM spectra. This approach is of great interest with respect to understanding histidine nitrogen ligation to the Mn cluster of PSII. An initial ESEEM investigation, probing ammonia binding to the Mn cluster, discovered a broad peak at approximately 5 MHz which was not associated with the ammonia nitrogen (Britt *et al.* 1989). Later, a 9 GHz ESEEM experiment using PSII core complexes from *Synechocystis* poised in the S_2 state showed a similar peak observed at approximately 5 MHz that disappeared with ^{15}N labelling of all the histidine residues (Tang *et al.* 1994). Further ESEEM experiments using PSII grown on media, in which only the π imidazole nitrogen of histidine is labelled with ^{15}N , have suggested that Mn cluster ligation occurs only through the τ nitrogen residue (Gilchrist 1996). Unfortunately, these low-frequency data suffered from poor modulation depth and, thus, the single broad peak gives little insight into the hyperfine and quadrupolar couplings.

To overcome the poor resolution of the 9 GHz data, studies of S_2 -state PSII samples isolated from spinach were extended by performing two- and three-pulse ESEEM experiments on a home-built pulsed EPR spectrometer (Sturgeon & Britt 1992) at four higher microwave frequencies (9.75, 10.96, 13.53 and 15.52 GHz). In order to minimize any g or hyperfine anisotropy effects, the spectra were recorded at a constant g -value of 1.98. The two- and three-pulse ESEEM frequency spectra are shown in figure 1*a,b*, respectively. In figure 1*a*, the high-frequency peak that tracks from 15 to 24 MHz is assigned to the matrix proton peak. The decrease in peak intensity can be attributed to the fact that the modulation depth is proportional to the inverse of the square of the magnetic field. The remaining low-frequency peaks (labelled with 'hash' and 'asterisk' symbols, respectively) in the two-pulse spectra of figure 1*a* show independent field-tracking trends. From linear least-squares analysis of this field-tracking behaviour (data not shown), the hash-labelled low-frequency peak tracks as a double quantum transition, while the higher frequency peak tracks as a single quantum transition. The three-pulse data in figure 1*b* show clear line-shape changes in the 5 MHz region as the magnetic field is increased; however, the peak position does not distinctively track as a single or double quantum transition and no signature quadrupolar transitions become apparent.

The multifrequency experiment just described highlights the inherent need for broadband ESEEM spectroscopy. Even with the ability to tune the microwave spectrometer over 6 GHz, the resultant data are still rather poorly resolved. This suggests that we are clearly well below the cancellation condition necessary for a strongly hyperfine-dominated ^{14}N coupling. In the rest of this article, we highlight the benefits of two intermediate frequency pulsed spectrometers and their increased sensitivity to nitrogen hyperfine couplings of globally labelled ^{14}N and ^{15}N PSII preparations. In addition, simulations of the dual-frequency ESEEM datasets will aid in the description of the histidine ligation to the Mn cluster.

Figure 1. Multifrequency (a) two- and (b) three-pulse ESEEM frequency-domain spectra of the S_2 state of PSII particles from spinach. In (a,b), the field positions corresponding to $g=1.98$ are labelled for each trace. The ν_{MW} frequencies were 9.749, 10.955, 13.530 and 15.517 GHz. In (b), the following τ values were used in the order of each spectra from top to bottom: 266, 237, 193 and 168 ns. All spectra were collected at 4.2 K with a repetition time of 5 ms.

3. K_a - AND Q-BAND ESEEM OF NATURAL ABUNDANCE ^{14}N -LABELLED PSII

The 31 GHz (K_a -band) experiments were performed on a high-power home-built pulsed spectrometer described elsewhere (Stich *et al.* 2007) and the Q-band were taken on a Bruker E580 pulsed spectrometer. Two- and three-pulse ESEEM experiments were performed at five magnetic field positions indicated by asterisks over the multiline absorption envelope, as shown in ESE-detected field-swept spectra in figure 2. The three traces (A)–(C) in figure 2 show the S_2 state of PSII from three separate samples as indicated in the figure legend. These light-minus-dark difference spectra, generated in order to isolate the light-induced $g=2$ multiline signal, are the result of subtracting the dark-adapted PSII particle spectra from the spectra obtained following 8 min white light illumination at 200 K. Each trace shows a narrow off-scale signal from the dark-stable tyrosine radical Y_D , as well as the partially resolved hyperfine couplings to the four ^{55}Mn nuclei. However, there are slight line-shape differences between the three traces. Trace (A)

Figure 2. Two-pulse K_a -band ESE field-swept 'light-minus-dark' normalized difference spectra of the S_2 -state multiline signal in PSII. The traces are offset for clarity. Trace (A) represents PSII particles from spinach treated with 3% ethanol. Trace (B) from PSII core complex of the cyanobacteria *Synechocystis* P6803 grown with natural abundance ^{14}N . Trace (C) from PSII core complex of the cyanobacteria *Synechocystis* P6803 grown in the presence of ^{15}N . The asterisks indicate the field positions in which two- and three-pulse experiments were performed. The arrow indicates the $g=1.98$ field position in which all the following data were collected. Instrument settings: ν_{MW} , 30.757 GHz; $\pi/2$ pulse, 30 ns; τ , 220 ns; magnetic field increment, 10 mT; repetition time, 5 ms; temperature, 4.5 K.

shows a Gaussian line shape, while traces (B) and (C) are slightly more truncated on the low-field side of Y_D . However, the integrated areas of the signals in PSII core particles from *Synechocystis* are within 10% of those from PSII particles prepared from spinach, indicating that the data are free of any residual cytochrome signals. Thus, the cause for the slight line-shape differences is unknown.

^{14}N *Synechocystis* ESEEM spectra obtained at K_a - and Q-band frequencies are shown in figure 3. Figure 3a,c shows the two- and three-pulse time-domain spectra, respectively. Figure 3b,d shows the corresponding ESEEM frequency domain after Fourier transformation of the respective two- and three-pulse time-domain spectra. As noted above, ESEEM was recorded at various field positions and τ values in the three-pulse data. However, the displayed datasets only show spectra taken at a constant g -value = 1.98, as indicated by the arrow in figure 2. The K_a - (bold line) and Q-band (thin line) time-domain spectra are strikingly similar. Both time-domain datasets show deep modulation relative to the previous X-band time-domain spectra (data not shown). This enhanced modulation clearly manifests itself in the well-resolved frequency-domain spectra of figure 3b,d. The K_a -band (bold line) two-pulse frequency-domain spectra of figure 3b show ^{14}N features as positive primary spectra

feature at 2.05, 6.58 and 13.84 MHz. Additionally, there are two negative features at approximately 4.51 and approximately 8.52 MHz flanking the positive 6.58 MHz peak. These negative peaks are combination peaks from fundamental frequencies ν_α and ν_β that appear as $\nu_\alpha \pm \nu_\beta$, $2\nu_{\alpha(\beta)}$, etc. (Kevan & Schwartz 1979). The two-pulse Q-band (thin line) frequency-domain spectra in figure 3b show similar positive and negative features as the K_a -band spectra, with all features above 2 MHz shifted to high frequency as a result of the higher microwave operating frequency. As shown in figure 3a, the echo amplitude decays approximately exponentially with increased τ . This decay is a result of T_2 or spin-spin relaxation processes, and thus makes two-pulse ESEEM experiments insensitive to low-frequency modulation components, as seen in the lack of resolution below 2 MHz in figure 3b.

The three-pulse time-domain data in figure 3c are not limited by T_2 relaxation processes, but rather by T_1 or spin-lattice relaxation. Thus, the carrier echo decays on a significantly longer time scale than that of the two-pulse experiment, making low-frequency modulations easier to detect. In addition to the enhanced low-frequency sensitivity, combination peaks do not complicate three-pulse experiments, in that only fundamentals are present for a single nucleus. However, the amplitudes of the fundamental nuclear transitions depend on the value of the first interpulse time τ , and thus certain frequency components may be suppressed for certain τ values. The K_a - (bold line) and Q-band (thin line) spectra in figure 3c show similar time-domain features with a modulation beat out to approximately 4 μs . The K_a -band frequency domain shows major spectral features at 0.88, 1.98, 6.90 and 14.00 MHz. The Q-band frequency-domain spectra show features at 0.90, 2.00, 7.14 and 14.65 MHz. Initial inspection of the two frequency-domain datasets suggests that the two low-frequency components are field independent and thus associated with ^{14}N quadrupolar transitions. The two higher frequency components show maxima that shift to higher frequency with higher microwave excitation frequency. In the limit of negligible quadrupolar effects, these slight shifts can be used as an initial assignment of single and double quantum transitions, with the assumption that single quantum transitions (ν_{SQ}) track as $\Delta\nu_I$ and double quantum (ν_{DQ}) transitions track as $2\Delta\nu_I$, where $\Delta\nu_I$ represents the difference in the ^{14}N Larmor frequency (ν_I) at two different spectrometer frequencies and matching magnetic fields. The 3.2 GHz frequency difference of the K_a - and Q-band spectrometers corresponds to a $\Delta\nu_{^{14}\text{N}} = 0.35$ MHz for a constant g -value, and thus is a reasonable difference to use in the field-tracking analysis. The K_a -band 6.90 MHz peak in figure 3d tracks to 7.14 MHz in the Q-band spectra. This difference of 0.25 MHz is within 30% of an expected single quantum shift of 0.35 MHz. Thus, this resonance is tentatively assigned as a single quantum transition in origin. The K_a -band 14.00 MHz peak shifts to 14.65 MHz in the Q-band spectra and thus shows a shift of 0.65 MHz, which is within 10% of the expected 0.70 MHz shift for a double quantum transition. Therefore, this high-frequency peak is tentatively assigned as

Figure 3. K_a - (bold line) and Q-band (thin line) ESEEM experiments of ^{14}N -labelled *Synechocystis* obtained at $g=1.98$, as indicated by the arrow in figure 2. (a) The two-pulse ESEEM time domain. (b) The corresponding two-pulse frequency domain. (c) The three-pulse ESEEM time domain. (d) The corresponding three-pulse ESEEM frequency domain. K_a -band instrument settings: ν_{MW} , 30.757 GHz; $\pi/2$ pulse, 10 ns; two-pulse $\Delta\tau$, 15 ns; three-pulse τ , 170 ns; three-pulse ΔT , 15 ns; repetition time, 5 ms; temperature, 4.5 K. Q-band instrument settings: ν_{MW} , 33.927 GHz; $\pi/2$ pulse, 16 ns; two-pulse $\Delta\tau$, 12 ns; three-pulse τ , 172 ns; three-pulse ΔT , 16 ns; repetition time, 5 ms; temperature, 4.5 K.

a double quantum transition in origin. Based on this field-tracking behaviour, the first interpretation of these datasets suggests that the peaks in figure 3d arise solely from ^{14}N interactions and the presence of two low-frequency peaks suggest that we are close to the cancellation condition for a nitrogen with a hyperfine coupling of approximately 6–8 MHz.

4. K_a - AND Q-BAND ESEEM OF ^{15}N -LABELLED PSII

To aid in the interpretation of the ^{14}N -labelled PSII data, PSII core samples were prepared from the cyanobacteria *Synechocystis* P6803, grown on ^{15}N -enriched media. The K_a - and Q-band two- and three-pulse ESEEM time- and frequency-domain spectra are represented in figure 4. Figure 4a shows the ^{15}N two-pulse time-domain spectra with the corresponding frequency-domain spectra in figure 4b. As with the two-pulse ^{14}N -labelled samples, the K_a - and Q-band time-domain spectra are quite similar in modulation depth; however, the frequency-domain spectra in figure 4b show distinct differences. The K_a -band (bold line) frequency-domain data show a strong peak at 9.50 MHz, which is roughly equivalent to twice the Larmor frequency ($\nu_{^{15}\text{N}}=4.78$) at a magnetic field of 1.108 T. Possible combination peaks (negative features) at 10.5 and 8 MHz are present as well, but the resolution is limited to above 2 MHz. The Q-band data in figure 4b (thin line) show distinctively different peaks than that of the K_a -band data. As with the K_a -band

data, there is a strong peak at 10.30 MHz, which is approximately $2\nu_{^{15}\text{N}}=10.58$ MHz at a magnetic field of 1.220 T. However, there is also a strong positive shoulder at approximately 9.4 MHz, two negative features at 7.80 and 11.30 MHz and a broad, less intense, positive feature at approximately 18 MHz. Again, the resolution is limited to features above 2 MHz. The complementary three-pulse time- and frequency-domain spectra are displayed in figure 4c,d, respectively. The time-domain spectra of figure 4c are similar at both operational microwave frequencies, with the K_a showing a more prominent low-frequency modulation. As with the two-pulse data, the three-pulse ESEEM frequency domain shows rather drastic differences at the two different operating microwave frequencies. The K_a spectra (bold line) show a high frequency peak at 9.7 MHz and a well-resolved low-frequency peak at 0.85 MHz. The Q-band spectra show a broad higher frequency peak at 10.16 MHz along with three low-frequency peaks: a sharp, intense peak at 0.15 MHz, with two broader features at 0.64 and 2.12 MHz.

Initial analysis of K_a spectra in figure 4d suggests that we are indeed close to the cancellation condition for an ^{15}N nucleus. In the limit of the exact cancellation for an $I=1/2$ nucleus, with a purely isotropic hyperfine coupling, a single peak at $2\nu_I$ would be present from the electronic spin manifold designated ν_+ , in which the nuclear Zeeman and hyperfine interactions sum together. Thus, this limit suggests that the isotropic

Figure 4. K_a - (bold line) and Q-band (thin line) ESEEM experiments of ^{15}N -labelled *Synechocystis* obtained at $g=1.98$, as indicated by the arrow in figure 2. (a) The two-pulse ESEEM time domain. (b) The corresponding two-pulse frequency domain. (c) The three-pulse ESEEM time domain. (d) The corresponding three-pulse ESEEM frequency domain. The K_a - and Q-band instrument settings are the same as described in figure legend 3, except that the three-pulse data were collected with a τ of 200 ns.

hyperfine interaction A_{iso} is equal to $2\nu_I$. As one moves above or below the cancellation limit, a peak from the electronic spin manifold in which the nuclear Zeeman and hyperfine interactions subtract from each other (ν_-) gains intensity. As such, the K_a 0.85 and 9.7 MHz peaks are assigned to the ν_- and ν_+ frequencies, respectively. The Q-band spectra are more complicated in that the presented dataset is for only one τ value. Two additional datasets at τ values of 172 and 212 ns (data not shown) show that the intensities of low-frequency peaks are rather τ dependent and, as such, should be summed in order to eliminate such effects. This additive analysis assigns the peak at 0.64 MHz with the highest intensity and is thus assigned to the ν_- transition. The results from the K_a - and Q-band data described above suggest an ^{15}N nucleus near the exact cancellation. In this limit, the peak positions of the high- and low-frequency components of each dataset allow for an initial estimate of the isotropic and dipolar hyperfine coupling, respectively. Therefore, we estimate an isotropic hyperfine coupling of 9–10 MHz and a dipolar hyperfine coupling of 0.85–1.2 MHz. These numbers serve as the basis for our initial simulation parameters, and, as such, a more detailed analysis of the data above is presented in §5.

5. ^{15}N ESEEM SIMULATIONS

To help facilitate an accurate determination of the hyperfine and quadrupolar couplings that give rise to the ^{14}N frequency-domain ESEEM spectra, we apply numerical simulations to first fit the ^{15}N ESEEM frequency domain. ^{15}N nuclei have a nuclear spin

$I=1/2$, and thus the datasets are not as complicated by the additional nuclear quadrupolar interaction, as with ^{14}N where $I=1$. With a good fit to the ^{15}N data, the hyperfine terms may be scaled as $^{14}\gamma/^{15}\gamma=0.71$ for the ^{14}N ESEEM simulations. Given the ground state $S=1/2$ spin state of the multiline signal of the S_2 state of PSII, we apply a MATLAB-written matrix diagonalization routine, based on the procedure of Mims (Mims 1972), to an $S=1/2$, $I=1/2$ system to evaluate the ^{15}N ESEEM spectra. The simulation assumes an axial hyperfine coupling as

$$A = \alpha_{\text{iso}} + \alpha_{\text{dip}}(3 \cos^2 \theta - 1),$$

where the dipolar (α_{dip}) contribution assumes the point-dipole approximation with a magnitude described by

$$\alpha_{\text{dip}} = (gg_n\beta_e\beta_n)\left\langle\frac{1}{r^3}\right\rangle.$$

In the above expression, g and g_n are the electron and nuclear g values, respectively; β_e and β_n are the Bohr and nuclear magneton, respectively; and r is the distance of the nucleus from the electron spin. The resulting time-domain spectra are multiplied by appropriate exponential functions to match the experimental data. Finally, Fourier transformation, analogous to that of the experimental data, is applied. It should be noted that these initial simulations assume only one nitrogen contribution and the goal is to simulate the K_a - and Q-band datasets with exactly the same spin Hamiltonian terms. Thus, the only differing

Figure 5. Simulations (dotted line) of K_a - (bold line) and Q-band (thin line) ESEEM experiments of ^{15}N -labelled *Synechocystis*. (a) The two-pulse ESEEM frequency domain with simulations below the experimental data. (b) The three-pulse frequency domain with the simulated spectra below the experimental data. The simulation parameters are $\alpha_{\text{iso}} = 9.68$ MHz and $\alpha_{\text{dip}} = 1.00$ MHz for all datasets. The simulations incorporate the instrument setting described in figure legend 4.

values in the simulation routine are that of the individual spectrometer settings.

In order to estimate the hyperfine coupling values for our simulations, we follow the analysis of our ^{15}N data in a manner described by Lai et al. (1989). For an $S = 1/2$, $I = 1/2$ system with an axial hyperfine coupling close to the exact cancellation, two peaks are expected: an intense low-frequency peak (ν_-) whose position is approximately $3|\alpha_{\text{dip}}|/4$ and a broader higher frequency peak (ν_+) with a position at $2\nu_I$. As the dipolar coupling constant can be estimated from the low-frequency resonance, the isotropic coupling constant can be estimated from the fact that, at the exact cancellation, $\nu_I \sim (2\alpha_{\text{iso}} + T)/4$. From the three-pulse K_a -band data, the peak at 0.85 is used to calculate an $\alpha_{\text{dip}} \sim 1.13$ MHz, which leads to an estimated α_{iso} of approximately 9 MHz. For the Q-band data, we use the 0.64 MHz peak to calculate an $\alpha_{\text{dip}} \sim 0.85$ MHz and α_{iso} of approximately 10 MHz. Given the differences in the estimated hyperfine coupling constants from the two datasets, we have varied both terms within their respective limits in our simulations. Thus far, the best fits to both datasets are shown in figure 5. In the interest of space, only the frequency-domain spectra are displayed.

Figure 5 shows frequency domain of the simulations and the experimental data for comparison, with the simulated spectra displayed as dotted lines directly below the experimental data. The hyperfine coupling values used in the simulated spectra are $\alpha_{\text{iso}} = 9.68$ MHz and $\alpha_{\text{dip}} = 1.00$ MHz. Figure 5a shows the simulated two-pulse spectra compared with the experimental frequency domain. Note that the K_a - and Q-band data are offset for clarity. Figure 5b shows the

simulated three-pulse data in the same manner as in figure 5a. As seen in figure 5a, we find relatively good agreement in simulating the high-frequency peak positions for the K_a - and Q-band two-pulse data; however, the overall line shapes are significantly different. With respect to the K_a -band three-pulse data in figure 5b, we again find good fits to both the high- and low-frequency peak positions, while the line shapes are rather distorted. In the Q-band data of figure 5b, we are able to fit the peak position of the high-frequency component; however, we have been unable to accurately simulate any of the lower frequency components without rather large shifts in the high-frequency component. We speculate that the line-shape differences between the simulated and experimental data are due to inaccuracies in properly fitting the modulation depth of the time domain (data not shown). Nevertheless, these calculated values serve as the first step in simulating the ^{14}N spectra as described in §6.

6. ^{14}N ESEEM SIMULATIONS

The calculated hyperfine values determined from the ^{15}N ESEEM spectra above are scaled, as described in §5, to begin the simulation of the K_a - and Q-band ^{14}N spectra. The nuclear quadrupolar Hamiltonian is added for the $I = 1$ ^{14}N nucleus. This new Hamiltonian term introduces two new parameters, e^2qQ and η , where the first component is the nuclear quadrupolar coupling constant and the second term is the asymmetry parameter. These two components further complicate the simulation method, as they introduce two more variables to manipulate in the simulations.

Figure 6. Simulations (dotted line) of K_a - (bold line) and Q-band (thin line) ESEEM experiments of ^{14}N -labelled *Synechocystis*. (a) The two-pulse ESEEM frequency domain with simulations below the experimental data. (b) The three-pulse frequency domain with the simulated spectra below the experimental data. The simulation parameters are $\alpha_{\text{iso}} = 7.4$ MHz, $\alpha_{\text{dip}} = 0.63$ MHz, $e^2qQ = 2.3$ MHz and $\eta = 0.8$ for all datasets. The simulations incorporate the instrument setting described in figure legend 4.

However, given the argument that the ^{14}N spectra appear to be near the exact cancellation limit, the low-frequency components of the data allow for initial interpretation of these values. We, again, assume the point-dipole approximation and proceed with our analysis using the scaled isotropic and anisotropic hyperfine couplings from the ^{15}N simulations to calculate an $\alpha_{\text{iso}} = 6.8$ MHz and $\alpha_{\text{dip}} = 0.71$ MHz for the ^{14}N hyperfine coupling constants. For the quadrupolar term η , two low-frequency peaks are clearly visible at approximately 1 and approximately 2 MHz, suggesting that η is approaching 1. The scaled isotropic hyperfine coupling and the field-tracking experiments in §3 allow for an initial estimate of e^2qQ of the order of 2–2.5 MHz. These four calculated values were used as the basis for our simulations, which allow for some deviation. The best simulations, thus far, are shown in figure 6a,b. Figure 6a describes the two-pulse ESEEM data and figure 6b the three-pulse data, with the simulated spectra displayed as dotted lines directly below the experimental data. The K_a - and Q-band simulations use exactly the same hyperfine and nuclear quadrupolar values with only differences related to the instrumental parameters, as described in the legend of figure 3. The following values were used in these simulations: $\alpha_{\text{iso}} = 7.4$ MHz; $\alpha_{\text{dip}} = 0.63$ MHz; $e^2qQ = 2.3$ MHz; and $\eta = 0.8$. The two-pulse data are in good agreement with the experimental with respect to overall peak positions. The simulations show an intense high-frequency peak and less-resolved mid-frequency combination peaks than the experimental data. In the three-pulse simulations, the simulations agree quite well with the mid- and high-frequency peak

positions; however, the low-frequency components are shifted to higher frequency. The line-shape differences, and the shifted low-frequency peaks in the three-pulse simulations, may be attributed to the limits in which these initial simulations were generated. Of important note, these simulations assume one nitrogen contribution, an axial dipolar hyperfine contribution, a relatively strong nuclear quadrupolar interaction and a high asymmetry parameter. As well, we do not rule out the possibility for another, more weakly coupled, nitrogen interaction. However, as a first approximation, our simulations suggest a single nitrogen interaction with a relatively strong hyperfine and nuclear quadrupolar interaction. Further simulations are underway to better fit the experimental data and properly describe the hyperfine and nuclear quadrupolar interaction.

7. DISCUSSION

The intermediate frequency ESEEM experiments described have shown significant sensitivity improvement to nitrogen couplings of the S_2 state of PSII. From the scaled isotropic hyperfine used in the ^{15}N simulations, we propose at least one moderately coupled ^{14}N interaction with α_{iso} of the order of 7 MHz. By equivalent scaling of the ^{15}N dipolar coupling to that of ^{14}N , we suggest a nitrogen distance of approximately 2.7 Å from the Mn cluster, in the point-dipole limit. We are currently refining our simulations to better model both the ^{15}N and ^{14}N datasets. As well, we have just begun further ESEEM and Q-band ESE-ENDOR experiments on *Synechocystis* samples globally labelled ^{15}N with the histidine residues labelled ^{14}N . With regard to the data presented, we tentatively assign the nitrogen

interaction to that of an imidazole nitrogen of a histidine residue directly coordinated to the Mn cluster of the OEC.

This argument for histidine ligation to the Mn cluster is in agreement with previous EPR and mutagenesis studies that show His332 is necessary for efficient photoassembly of the Mn cluster (Campbell *et al.* 2000), as well as proper S-state turnover (Chu *et al.* 1995; Debus *et al.* 2000; Allahverdiyeva *et al.* 2004). Further evidence for His332 coordination is provided by ESEEM studies of the D1-H332E mutant (Debus *et al.* 2001). This study showed that the approximately 5 MHz feature in the 9 GHz two-pulse time-domain ESEEM spectrum essentially disappears in the D1-H332E mutant, providing evidence that D1-His332 ligates Mn. In addition to the above mentioned discussion, X-ray crystallographic data of PSII have proposed histidine residues near the electron density pocket of the Mn cluster (Ferreira *et al.* 2004; Loll *et al.* 2005). While the exact structure of the Mn cluster is still unknown, recent polarized X-ray absorption fine structure (EXAFS) and X-ray diffraction experiments were done to reassess the proposed structure of the Mn cluster (Yano *et al.* 2006). Even in this new proposed structure of the Mn cluster, possibly two histidine residues from the D1 polypeptide (His332 and His337) lie within the first coordination sphere.

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Discussion

C. Dismukes (*Princeton University, USA*). Have the new EPR spectrometers provided any information on how

the ^{55}Mn nuclear quadrupolar interaction changes up coordination changes?

D. Britt. Not yet, we've not yet focused on the high-frequency ENDOR region with the new instruments, though we hope to soon.

A. B. Doust (*Free University, Amsterdam*). Time-resolved data were presented and the oscillations Fourier transformed to show peaks in the frequency domain. My question is, what information can be gained from the time-domain data?

D. Britt. We typically simulate the time-domain data, particularly to match the amplitudes of modulation, which can be used to quantify the number of coupled nuclei. This is rather similar to EXAFS, where one typically analyses the k-space data rather than the transform. In both cases, there is a gap in the initial dataset (here time domain due to pulse ring-down dead time), so analysing in the Fourier space involves some pitfalls as that frequency-domain data include the effects of dead-time reconstruction.

H. Dau (*Freie University, Berlin, Germany*). Will it be possible to use the presented EPR techniques to address the oxidation state of the Mn ion which (likely) is coordinated to D1-His332?

D. Britt. Not trivially, but perhaps through detailed electronic structure calculations as methods improve. Targeted observables here could include both the hyperfine and the ^{14}N quadrupolar interaction.