Studies on 100% turnover higher plant photosystem II, as revealed by 55Mn EPR and Davies ENDOR

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By

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Declaration

The thesis comprising of my original work is presented, except where otherwise acknowledged, and has not been submitted in whole or part for a degree in any University. It was completed in the Research School of Chemistry at ANU during the period March 2009 to July 2015.

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Publications

The following works have been published during the course of the PhD study:


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Abstract

Photosystem II or PS II, found in oxygenic photosynthetic organisms such as cyanobacteria or higher plants, is the catalyst for the most energetically demanding reaction in nature, the oxidation of water to molecular oxygen and protons. The water oxidase in PS II contains a Mn₄Ca cluster (oxygen evolving complex, OEC), whose catalytic mechanism, despite extensive investigation, remains unresolved. The precise oxidation levels of the manganese is especially important in understanding the real catalytic mechanism of the OEC. Many experimental techniques, such as EPR and ENDOR, have been adopted in historical studies, and also due to the more recent development in semiconductors, a higher level of computational analysis and simulation became available to study the system in theory. Here is the work completed to provide the first $^{55}\text{Mn}$ pulsed ENDOR studies on the $S_2$ state multiline spin $\frac{1}{2}$ centre of the oxygen evolving complex (OEC) in Photosystem II (PS II), at temperatures below 4.2 K. These were performed on highly active samples of spinach PS II core complexes, developed previously in the laboratories using specific preparation procedure and experimental techniques to achieve 100% turnover rate, for photosystem spectroscopic use, at temperatures down to 2.5 K. Under these conditions, previously hindered observation, by relaxation effects, of most of the manganese ENDOR resonances from the OEC coupled Mn cluster are suppressed.$^{55}\text{Mn}$ ENDOR hyperfine couplings ranging from 50 to 680 MHz are now seen on the $S_2$ state multiline EPR signal. These, together with complementary high resolution X-band CW EPR measurements and detailed simulations, reveal that at least two and probably three Mn hyperfine couplings with large anisotropy are seen, indicating that three Mn$^{\text{III}}$ ions are likely present in the functional $S_2$ state of the enzyme. This suggests a low oxidation state paradigm for the OEC (mean Mn oxidation level 3.0 in the $S_1$ state) and unexpected Mn exchange coupling in the $S_2$ state, with two Mn ions nearly magnetically silent. Our results rationalize a number of previous ligand ESEEM/ENDOR studies and labelled water exchange experiments on the $S_2$ state of the photosystem.
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>$P_{680}$</td>
<td>primary electron donor of photosystem II</td>
</tr>
<tr>
<td>$P_{680}^*$</td>
<td>highly activated excited state of $P_{680}$.</td>
</tr>
<tr>
<td>$P_{680}^+$</td>
<td>strong oxidising agent.</td>
</tr>
<tr>
<td>ML</td>
<td>multiline signal</td>
</tr>
<tr>
<td>PSII</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>PSI</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Chl</td>
<td>chlorophyll</td>
</tr>
<tr>
<td>$Q_A$</td>
<td>primary plastoquinone acceptor</td>
</tr>
<tr>
<td>$Q_B$</td>
<td>secondary plastoquinone acceptor</td>
</tr>
<tr>
<td>Tyr$_D$/Y$_D$</td>
<td>tyrosine residue located in D2 protein</td>
</tr>
<tr>
<td>Tyr$_D}$/Y$_D$</td>
<td>tyrosine residue located in D2 protein</td>
</tr>
<tr>
<td>OEC</td>
<td>oxygen-evolving complex of photosystem II</td>
</tr>
<tr>
<td>WOC</td>
<td>water oxidizing complex of photosystem II</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>CW</td>
<td>continuous wave</td>
</tr>
<tr>
<td>DFT</td>
<td>Density Functional Theory</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>XANES</td>
<td>X-ray absorption near-edge structure</td>
</tr>
<tr>
<td>EXFAS</td>
<td>Extended X-ray absorption fine structure</td>
</tr>
<tr>
<td>RC</td>
<td>Reaction Centre</td>
</tr>
<tr>
<td>Mn</td>
<td>Manganese</td>
</tr>
<tr>
<td>$\mu_e$</td>
<td>Magnetic moment of an electron</td>
</tr>
<tr>
<td>$H_0$</td>
<td>Steady magnetic field</td>
</tr>
<tr>
<td>$H_1$</td>
<td>Oscillating magnetic field</td>
</tr>
</tbody>
</table>
CP43, CP47  proximal antenna pigment-protein complexes of PSII
D1, D2  Proteins that bind the cofactors of the PSII reaction centre
LHCII  Light harvesting complex of photosystem II
EG  Ethylene glycol
DMCU  3-(3, 4-dichlorophenyl)-1, 1-dimethylurea
pPBQ  phenyl-p-benzoquinone
S  spin angular momentum vector
I  orbital angular momentum vector
$a_i$  Hyperfine coupling constant
$m_s$  Spin magnetic quantum number
$m_l$  Orbital magnetic quantum number
D  Zero field splitting tensor
$\zeta$  Spin-orbit coupling constant
$\beta_e$  $\frac{e\hbar}{2mc} = $ Electron Bohr magneton.
$\beta_N$  $\frac{e\hbar}{2Mc} = $ Nuclear magneton
L  operator for orbital angular momentum
Q  quadrupole moment tensor
$J_i$  Heisenberg exchange constant
$\rho_i$  projection coefficient
ESR  Electron spin resonance
XRD  X-ray diffraction
Cytb_{559}  Cytochrome b559-intrinsic component of PSII
Cytb_{6f}  Cytochrome b_{6f}
S-state  redox intermediate states (denoted S1, S2, S3, S4 and S0).
XAS  X-ray absorption spectroscopy
XES  X-ray emission spectroscopy
ESEEM  Electron spin echo envelope modulation
His332  ligating histidine
FTIR  Fourier transform infrared spectroscopy

\( g_N \)  Nuclear \( g \) factor.

\( g_e \)  Electron \( g \) factor.

\( k \)  Boltzmann constant=\( 1.38065 \times 10^{-23} \text{JK}^{-1}. \)

\( T \)  Temperature (Kelvins).

\( C \)  is a measure of true signal intensity at low power limit.

\( P \)  microwave power

\( P_{1/2} \)  half-saturation microwave power

\( T_1 \)  spin lattice relaxation time.

\( T_2 \)  spin-spin relaxation time.

\( a_{iso} \)  Fermi contact hyperfine coupling constant.

ENDOR  Electron nuclear double resonance

ZFS  Zero field splitting.

LHC  Light harvesting complex

HPLC  High-performance liquid chromatography

SI  Signal Intensity.
Photosynthesis is the key process of providing the energy source for life, in particular carbohydrates, for both cyano-bacteria and higher plants. Alternatively, it provides energy for all living species on earth through the food cycle, by transforming solar change power into biochemical energy.

Photosynthesis is also one of the most crucial reasons that non-photosynthetic life exists. It has reshaped our atmosphere, which was composed mainly by nitrogen and carbon dioxide, for approximately 2.5 billion years. This “earliest atmosphere” could not support oxygen breathing life forms, such as animals. Photosynthesis, with its carbon dioxide fixation ability, gave rise to our atmosphere today, which has 78% nitrogen, and 21% oxygen, through the following reaction.

Chemical Equation (1.1.1):

\[
2n \text{H}_2\text{O} + n \text{CO}_2 + x \text{hv} \rightarrow [\text{CH}_2\text{O}]_n + n \text{H}_2\text{O} + n \text{O}_2
\]

A further vital importance is the formation of fossil fuels, etc., through transformation of decaying plant material by geological processes. As indicated in Figure 1.1, roughly 60-70% of the earth surface is covered with various degrees of photosynthetic reactions. Hence some consider it to be the most important biological process.

Photosynthesis does not only have academic importance, but is also very closely connected to the solution of the energy crisis, which may occur in the foreseeable near future.
Figure 1.1 the global distribution of photosynthesis. The green, purple and blue colours indicate strong photosynthetic presences.

(Image source: http://upload.wikimedia.org/wikipedia/commons/4/44/Seawifs_global_biosphere.jpg)

It took nature 2.5 billion years to have photosynthetic organisms evolve to their form today: they contain a series of nano scale energy converting protein complexes composed of over 100,000 atoms each, with very efficient light harvesting antennae. Photosynthesis is fundamentally associated with two most promising forms of future energy solutions: bio-electronic cells (fuel cells), and the mass production of hydrogen by electrochemical means. Both approaches are clean and recyclable. No other known system, whether it is natural or artificial, has the same capability. Is no surprise that it attracts the attention of so much academic study [1].
There are four key enzymes involved in oxygenic photosynthesis: Photosystem I and II (PS I & II), the cytochrome b₆f complex and ATP synthase. All four are embedded in the thylakoid membrane, which sits inside the chloroplast, with an inner region known as the “lumen”, and an outer region the, “stroma” [2], as illustrated in Figure 1.2.1.

Figure 1.2 an illustration of the photosynthetic process.

There are a number of linked reactions associated with this complex system, all centred on efficient light-harvesting and energy exchange/transformation. The two photosystems, II and I, contain light-driven redox centres. They have antenna complexes that contain protein bound pigments, principally chlorophylls, xanthophylls, and carotenoids. PS II uses the xanthophyll cycle, which occurs at its outer antenna light-harvesting complex II (LHCII), to enzymatically remove the epoxy groups from xanthophylls to lose excess energy arising
through (high) light absorption, as a means of photo-protection to the whole system [3]; The cytochrome b$_6$f complex acts as an intermediate in the transport of electrons from the plastoquinol formed by PS II action to plastocyanin, a mobile (soluble) protein, which donates electrons to PS I. In this process protons are moved across the thylakoid membrane, increasing the proton gradient. The ATP synthase, at the end of the process, generates adenosine triphosphate (ATP) as the consumable bio-energy source for the biological system [4].

This -nature’s most important energy transferring process- starts from the photo activation of electrons derived from the catalysed splitting of water within PS II, which also releases mobile protons during the splitting process, for use in latter reactions. It holds the key of unlocking the mystery of these reactions; therefore, PS II is the primary interest of this work.
1.3 PHOTOSYSTEM II

1.3.1 Cofactors of PS II

The primary function of photosystem II is the catalytic, light-driven water oxidation reaction. The PS II complexes in higher plants and cyanobacteria are very similar in their main protein scaffold, with some differences in the inner cofactors. In higher plant PS II, our primary interest, the essential cofactors required for its functionality are all situated in the D1 and D2 protein subunits, which are very similar overall. Some cofactors in the D2 protein do not now participate in the normal turnover reaction, probably due to evolutionary refinement of function over many millions of years. Only the plastoquinone Q_B binding pocket retains full function in D2, while the tyrosine Y_D amino acid residue also participates at particular stages, but not in the continuous water splitting process [4]. Figure 1.3.1 shows the cofactors in the D1 and D2 subunits.

The D1 unit contains, or partially contains, the following cofactors: the Oxygen Evolving Complex (OEC, also called the water oxidising complex, WOC), which is directly responsible for the oxidisation of water; a chlorophyll containing photochemical reaction centre, P680; a redox active tyrosine \( Y_3 \); a primary electron acceptor pheophytin_\(D_1 \), and a secondary electron acceptor, plastoquinone Q_A , which is also the final electron acceptor in D1 before the electrons are transferred through a non heme iron centre to the mobile electron carrier, plastoquinone Q_B . Together this forms the “electron pathway” in PS II [5], as partially indicated in Figure 1.3 as well.
1.3.2 Electron acceptor and donor pathways

P680 is readily photo-excited by the light harvested from the antenna complex. It photo-oxidises and transfers one electron to the primary acceptor pheophytinD1, then this electron is passed onto the secondary electron acceptor QA, which is now reduced to QA-. A plastoquinone QB from the QB binding pocket, which is now the only cofactor that retains full functionality in D2, oxidises QA- back to QA, through an intervening Fe2+ bridge that accelerates the process (QA-Fe2+-QB). The QB- so formed is ultimately a double electron carrier, which when the above process has occurred again, is further reduced to QB2-.
binds two free protons from the stroma to become a quinol alcohol, H₂Q₈. Then this mobile molecule leaves the Q₈ binding pocket and is replaced by another free Q₈ from the surrounding membrane environment, after which the whole process repeats. This is called the acceptor pathway [6].

The highly oxidising P680⁺ cation takes one electron from the nearby tyrosine, Y₂, to reduce itself back to a stable state. The oxidised Y₂⁺ then reduces itself by extracting one electron from the OEC, a 4-manganese-1-calcium complex that will provide four electrons by splitting two water molecules per reaction cycle. This is explained in detail below [5b]. The total process is known as the donor pathway.

1.3.3 Oxygen Evolving Complex

The main component of the OEC is a Mn₄CaO₄ cluster, in which three of the four manganese ions and one calcium ion are all linked through oxo-bridges, forming a cubane-like structure. One oxo and one carboxylato bridge also connect this cubical cluster to the other manganese. This has been confirmed by several crystal structures, with various levels of resolution, published in recent years [7]. Figure 1.4 shows the 2.9Å crystal structure of Loll et al.
The more recent 1.9Å Umena structure [7e]) shows the oxo-bridges linking the Mn, though there are still questions related to the precision of the structure and possible influences of radiation damage during data collection [8]. These crystal structures correspond, nominally at least, to the ‘dark stable’ state.

1.3.4 The Kok cycle

In 1970, Kok et al. first described the mechanism of the OEC as proceeding through four electron transfer steps (forming the so-called S state intermediates), which consist of four stable states (S₀, S₁, S₂, S₃) and a transient, short-lived ‘final’ state (S₄) [9]. The subscript
numbers refer to the number of stored oxidising equivalents in the catalytic centre. Water molecules remain freely exchangeable with the OEC up to S₃ [10], and the final oxidation of two water molecules to di-oxygen occurs in a concerted, four-electron step.

All S states exhibit various degrees of EPR sensitivity under controlled experimental conditions, with the S₀ and S₂ being the most significant. These two states have odd numbers of unpaired electrons in this exchange coupled cluster, with net spin ½ ground states arising from a predominantly anti-ferromagnetic coupling of the Mn ions [11]. The S₂ state EPR signal, ‘multiline’, was first reported by Dismukes et al. in 1981 [12], and the S₀ state signal discovered 16 years later, by two groups simultaneously [13].

1.3.5 The low and high Mn oxidation states paradigms

Despite extensive study on the OEC, the full catalytic mechanism underlying the water oxidation reaction remains unresolved. Both the location of substrate water binding sites and the detailed redox changes undergone by the Mn in the site are contentious [14]. It is clear that elucidation of the water splitting mechanism in the OEC will require, amongst other things, that the oxidation states of the Mn ions throughout the reaction cycle be properly identified, as these set the ‘chemical stage’ upon which possible mechanistic pathways may operate. The reaction is known to proceed under very tight energy constraints, with the mid-point potential of the Y₂/Y²⁺ couple being 1.0-1.1 V [15], while the mean potential per electron for concerted water oxidation at pH ~ 6 is close to 0.9 V. Thus the system operates near to the thermodynamic limit [16].

Photo-assembly [17] and Mn XANES measurements (eg. see [18] on a variety of PS II preparations indicate clearly that the mean Mn oxidation states in the functional cluster (i.e.
in states $S_0$ and above) are significantly higher than II. This then dictates that the formal oxidation state in $S_1$ is $\text{Mn}^{III}\text{Mn}^{III}_2 \text{Mn}^{IV}$ or $\text{Mn}^{III}_2\text{Mn}^{IV}_2$, or combinations equivalent to these, with mean oxidation levels in the other $S$ states determined by adding or removing electrons. These have been labelled the ‘low and high’ oxidation state paradigms respectively [19]. Data from a range of spectroscopic techniques applied to PS II, which bear upon this matter, have recently been reviewed, but this did not consider EPR [20]. The question is currently contentious and in the low and high paradigms, $S_2$ is $\text{Mn}^{III}_3\text{Mn}^{IV}$ or $\text{Mn}^{III}\text{Mn}^{IV}_3$, respectively. Figure 1.5 [19] shows the Mn oxidation states in the low and high paradigms.

At present, the high oxidation state assignment is generally favoured, based principally on empirical interpretation of Mn X-ray absorption spectroscopies applied to PS II in the $S_1$ state and $S$ states generated by single turnover flash advance. [21]. However, recent theoretical work has shown, using a new Time Dependent DFT (TDDFT) approach, that the results from the most extensively used X-ray absorption technique, Mn K edge analysis, are consistent with the low oxidation state paradigm [22], when metal ligand environment effects are computationally accounted for.
Figure 1.5 the low and high Mn oxidation states paradigm.
CHAPTER II ELECTRON PARAMAGNETIC RESONANCE
2.1 BACKGROUND OF EPR

2.1.1 Introduction

Nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR, also called electron-spin resonance, ESR) are fundamentally similar to each other. They were both developed about 50 years ago and operate by the same basic principals. They have both contributed greatly to our modern discoveries in physics, chemistry, biology, medicine, etc.

Most molecules do not possess unpaired electrons, which is the fundamental requirement for an EPR experiment. Hence by comparison, NMR is more widely used in current scientific research. However, this limitation is a double-edged sword, which EPR also benefits from, as conventional chemical solvents and media do not give rise to EPR signals.

With instrumental technology advancement, the once relatively limited continuous-wave EPR (cw-EPR) technique has not only increased in range of microwave frequency; from S-band (~3 GHz) to X-band (~9 GHz), Q-band (~35 GHz), even W-band (~95 GHz), but has also been greatly enhanced by the application of pulse techniques (both microwave and radio-frequency, as for NMR). As a consequence, a wide range of powerful EPR based spectroscopic tools is now available, including electron-nuclear double resonance (ENDOR), electron spin-echo envelope modulation (ESEEM), hyperfine sub-level correlation (HYSCORE), and electron-electron double resonance (ELDOR), etc. [23]

Thus, EPR has become an increasingly powerful experimental tool, with specific strength in the study PS II, an electron transfer complex. Here, a brief introduction to the basics of NMR is also given, as it will be needed in later chapters dealing with ENDOR experiments.
2.1.2 Spin

Sub-atomic particles, such as electrons, protons and neutrons are associated with a fundamental physical property called “spin”. Any nucleus with an odd mass number, such as $^1\text{H}$ and $^{13}\text{C}$, possesses a net nuclear spin from the overall spin combination of its internal particles. Even mass number nuclei may also possess net spin under some circumstances, e.g. $^2\text{H}$, $^{14}\text{N}$, etc. Spin is a purely quantum-mechanical phenomenon, derived from the intrinsic form of angular momentum carried by elementary particles.

The spin can be determined as the following: 1. A nucleus that carries an even number of protons and an even number of neutrons, has a net spin of zero; 2. An odd number of neutrons and an odd number of protons sum up to an integer spin, such as 1, 2, 3, etc. 3. If the total number of the neutrons plus protons is an odd number, the corresponding nucleus possesses spin of a half-integer, such as $1/2$, $3/2$, $5/2$. 4. A free electron always carries a spin of $1/2$, known as a fermion, so the number of unpaired electrons in a system determines its electron spin [24].

2.1.3 The magnetic moment

In a nuclear system, the spin angular momentum vector is described as $\mathbf{I}\hbar$, whereas $\mathbf{I}$ is the nuclear spin operator, and the magnitude of spin, $I$, is an integral number multiplied by $1/2$. The constant $\hbar$ is Planck’s constant divided by $2\pi$, which takes a value of $1.055\times10^{-34}$ J-s, and establishes the units of the system ($I$ is dimensionless).

To mathematically describe the magnetic properties of a nucleus, we must take into account both the spin and electronic charge that the nucleus possesses. In combination these grant
the nucleus a magnetic moment $m_N$ that is directly proportional to the value of the spin, and this is described by Equation (1).

$$m_N = \gamma_N \hbar I = g_N \beta_N I$$  \hspace{1cm} \text{Equation (1)}

Here, $\gamma$ is the gyromagnetic ratio of the nucleus, which has units of rad\cdot s^{-1}\cdot G^{-1}. As shown, the magnetic moment can also be expressed as the product of $\gamma$, the nuclear $g$ factor and $\mu$ the nuclear magneton, which is described by Equation (2).

$$\beta_N = \frac{e\hbar}{2Mc}$$  \hspace{1cm} \text{Equation (2)}

Here $e$ is the charge and $M$ the mass of the proton, with $c$ being the speed of light. The $g_N$, $g_N$ and $I$ quantities are characteristic values, which may differ from nucleus to nucleus.

The magnetic moment of an electron $m_e$ can be expressed similarly as Equation (3).

$$m_e = -g\beta S$$  \hspace{1cm} \text{Equation (3)}

Here $g$ is a constant known as the electron $g$ factor, $\mu$ the Bohr magneton and $S$ the electron spin. The equation is negative because the Bohr magneton, written as $\mu = e\hbar/2mc$, now has $e$ as the electron charge that is negative, while $m$ is the electron mass.
2.2 SPIN HAMILTONIAN

2.2.1 The Zeeman Effect and spin Hamiltonian

The spin Hamiltonian is the energy operator, which theoretically describes EPR [25]. As far as this work is concerned, it includes the following components: the nuclear Zeeman ($H_{NZ}$), the electron Zeeman ($H_{EZ}$), the hyperfine couplings ($H_{HF}$), and the nuclear quadrupole interactions ($H_{QP}$). Here, we describe the first two terms to provide a theoretical snapshot of EPR, and then examine the remaining two later.

The allowed nuclear spin states of each nucleus are quantized along a particular direction in space, conventionally taken as the z-direction, described by the spin quantum number, $m_i$. For a proton, $^1H^+$, with $m_i=1/2$, or $-1/2$, each $m$ value represents an eigenstate of the spin system with a corresponding eigenenergy. The two states are degenerate in energy without an external magnetic field being present.

Phenomenologically, once an external magnetic field, $B$, exists with certain magnitude and direction, the two eigenstates are no longer degenerate due to the interaction of the magnetic moment of the spin system with this external field. Such an effect is known as the Nuclear Zeeman Effect. It can be described by its spin Hamiltonian as Equation (4):

$$H_{NZ} = -g_N\beta_N B \cdot I$$  \hspace{1cm} \text{Equation (4)}

If (as normally) the $B$ vector defines the z spatial direction, then $B \cdot I = Bm_i$ and the normal selection rule for simple radiation induced transitions is $\Delta m_i = \pm 1$. Solution of the spin Hamiltonian gives the eigenenergies of the corresponding states and together with the selection rule, the energy of a particular transition, $E$, can be written;
\[ \Delta H_{NZ} = -g_N \beta_N B(\Delta m_l) = E = h\nu \]

Equation (5)

Where \( h \) is again Planck’s constant, and \( \nu \) is the frequency of the electromagnetic radiation that would excite the transition, or be emitted as radiation between the two no-longer-degenerate eigenstates, for example the energy difference between states \( m_l = 1/2 \), and \(-1/2\) of a proton (\( \Delta m_l = 1 \)).

The Electron Zeeman Effect: this is analogous to Equation (4). Equation (7) shows the generalised electron Zeeman term:

\[ H_{EZ} = \beta B \cdot \tilde{g} \cdot S \]

Equation (6)

Here in this more generalised form, \( \tilde{g} \) is a symmetric tensor, used to represent the anisotropic interaction typically occurring in ions, molecules, that differs from a free electron g-factor (its scalar value is 2.0023) and both \( B \) and \( S \) are vectors. The whole tensor interaction can be expressed in full as Equation (7):

\[
\begin{bmatrix}
B_x & B_y & B_z \\
g_{xx} & g_{yy} & g_{zz} \\
g_{xy} & g_{yx} & g_{yz} \\
g_{xz} & g_{zx} & g_{zz}
\end{bmatrix}
\begin{bmatrix}
S_x \\
S_y \\
S_z
\end{bmatrix}
\]

Equation (7)

Two Zeeman effects combine energetically in separating the eigenstates. For example, a hydrogen atom \(^1\text{H}\) has 1 proton plus 1 electron, giving rise to four separate eigenstates: \( |m_S, m_l> \), where both \( m_S \) and \( m_l \) can take the values of \( 1/2 \) and \(-1/2\). Figure 2.1 provides an illustration of the combination between the two Zeeman effects, in first order [23].
2.2.2 The hyperfine coupling

An electron with an electronic magnetic moment may interact with a nearby nuclear magnetic moment, through the so-called “magnetic hyperfine interaction”. This effect can be understood as arising from magnetic interaction of the nuclear magnetic moment with the magnetic field at the nucleus that is set up by the electron cloud, or alternatively arising from the interaction of the electron magnetic moment with the magnetic field that is set up by the nuclear magnetic moment [26].
The hyperfine interaction typically consists in total of an isotropic component, or (Fermi) ‘contact interaction’, having the same magnitude interaction along each spatial direction and an anisotropic component, with in general a different hyperfine interaction along different spatial directions. The latter typically arises from the electron orbital averaged value of the (point) dipole-dipole magnetic interaction between the unpaired electron spin and the nucleus [27]. It is normally traceless, i.e. its isotropic component is zero.

The Hamiltonian of such electron-nuclear interaction would involve both the electron spin $S$ and the nuclear spin $I$. Equation (8) shows the Hamiltonian of the isotropic hyperfine coupling, and the anisotropic part is shown in Equation (9).

\[
H_{HF}^{iso} = h a S \cdot I \tag{8}
\]

\[
H_{HF}^{aniso} = h S \cdot \mathbf{\tilde{A}} \cdot I \tag{9}
\]

Here, $a$ is the isotropic hyperfine coupling constant, which has the dimensions of frequency ($ha$ is energy) and is proportional to the squared amplitude of the electronic wave function at the nucleus, shown in Equation (10).

\[
a = \frac{8\pi}{3} g_e \beta_N g_e \beta_N |\Phi(0)|^2 \tag{10}
\]

$\mathbf{\tilde{A}}$ is the (traceless) hyperfine tensor that has units of frequency. It is expressed as Equation (11):
Hence, the complete Hamiltonian for the hyperfine interaction would be as Equation (12):

\[
H_{HF} = H_{HF}^{iso} + H_{HF}^{aniso} = \hbar \alpha \mathbf{S} \cdot \mathbf{I} + \hbar \mathbf{S} \cdot \mathbf{\tilde{A}} \cdot \mathbf{I} 
\]

Equation (12)

Once the hyperfine interaction is introduced, the EPR spectrum is split further (from the Zeeman splitting), as illustrated in Figure 2.2.2 using a hydrogen atom as an example [23, 26, 27]:

Figure 2.2 The combined effects of the nuclear and electron Zeeman interactions, and the hyperfine coupling on the eigenstates and eigenenergies of the hydrogen atom spin system.
Figure 2.2 also indicates the spin allowed transitions for EPR (red arrows) and NMR (blue arrows) respectively, subject to the simple selection rules $\Delta m_{i,S} = \pm 1$. Both the electron only and proton only transitions are now ‘split’ in energy due to the hyperfine interaction. This is only a very simple case for a hydrogen atom, but the Mn ions that interests us in this work are more complicated, especially when we have four manganese interacting with each other, which all exhibit these magnetic properties. One would expect to see many lines that may overlap between the four manganese, in the “multiline” signal, which we will later explain in more detail.

2.2.3 The quadrupole interaction

Nuclei that have a spin greater than or equal to 1, i.e. $I \geq 1$, such as $^{55}$Mn with a nuclear spin of 5/2, possess a nuclear electric quadrupole moment as well as a magnetic moment. This quadrupole moment may interact with any non spherically symmetric component of the local electric field (from the electrons) near the nucleus. If this quadrupole interaction is weaker than the hyperfine interaction, which is often the case, it does not to a first order approximation affect the experimental EPR spectra. However, it does make a difference in the ENDOR spectra, causing further splitting according to the Hamiltonian of the quadrupole term, shown in Equation (13).

$$H_Q = \hbar \cdot \vec{Q} \cdot \vec{I}$$

Equation (13)
Here \( \tilde{Q} \) (frequency units) is the quadrupole coupling tensor, expressed as Equation (14):

\[
\begin{pmatrix}
Q_{xx} & Q_{yx} & Q_{zx} \\
Q_{xy} & Q_{yy} & Q_{zy} \\
Q_{xz} & Q_{yz} & Q_{zz}
\end{pmatrix}
\]  
Equation (14)

2.2.4 The complete Hamiltonian

The complete Hamiltonian for a single electron-nuclear system would then be a combination of the terms above, shown in Equation (15) and (16):

\[
H = H_{\text{NJ}} + H_{\text{EZ}} + H_{\text{HF}} + H_Q
\]  
Equation (15)

\[
H = -g_e\beta B \cdot I + \beta B \cdot \tilde{g} \cdot S + h a S \cdot I + h S \cdot \tilde{A} \cdot I + h I \cdot \tilde{Q} \cdot I
\]  
Equation (16)
2.3 CALCULATING THE HAMILTONIAN

2.3.1 Introduction to methods of calculation

To start the calculation, we know that if an operator C and a non-vanishing vector $|u\rangle$ are related as Equation (17):

$$C|u\rangle = k|u\rangle$$  \hspace{1cm} \text{Equation (17)}

Where $k$ is a constant, so that the operation C sends $|u\rangle$ into a numerical multiple of itself, then $k$ is called the eigenvalue of C, and $|u\rangle$ is called the eigenvector of C. $k$ and $u$ are said to ‘belong’ to each other [28].

A Hamiltonian is the operator corresponding to the complete energy of a system. When we apply the Hamiltonian operator to a wave function that represents the quantum system in a particular state, $|\psi\rangle$, i.e. an eigenvector or an eigenstate, we are able to obtain the quantised energy, i.e. energy eigenvalue, of that eigenstate, i.e. the eigenenergy, shown in Equation (18):

$$H|\psi\rangle = E|\psi\rangle$$  \hspace{1cm} \text{Equation (18)}

For an EPR sensitive system, if we used the proton again as an example, we would obtain 4 corresponding eigenenergies, which would contain 2 spin-allowed EPR transitions (shown in Figure 2.2), hence we should observe 2 EPR signals in an ideal experiment. Thus, we would be able to computationally simulate the experimental EPR spectrum of the proton, by solving the Hamiltonian and computing the eigenenergies. This explains a simplified case of “simulation”.
2.3.2 The eigenstates of the Mn$_4$ system (matrix construction)

Before addressing the method of calculation, we must first construct the matrix representing the Hamiltonian of the Mn$_4$ system. The system of interest, the $S_2$ state OEC has a net electron spin of $\frac{1}{2}$ arising from a net antiferromagnetic coupling of the ions, that is well established, following its discovery by Dismukes rt al (ref) [29]. The nuclear spin of a manganese atom/ion is 5/2, so the spin system of interest contains four nuclei, each with spin of $l=5/2$ and a net unpaired electron with spin $S=1/2$. Hence we have a spin system of “Mn$_4$+e”.

Equation (19) shows the Hamiltonian applying on the “Mn$_4$+e” spin system:

$$\hat{H}\psi_e, \psi_1, \psi_2, \psi_3, \psi_4 = E\psi_e, \psi_1, \psi_2, \psi_3, \psi_4$$ 

Equation (19)

Here $\psi_e$ is the wave-function (labelled) of the electron; and $\psi_n$ (n = 1, 2, 3, 4) is the wave-function labelling of the corresponding nuclei. $E_l$ (l = 1, 2, 3, ...) is the eigenenergy of the corresponding eigenstate obtained through applying the Hamiltonian operation. Both components labelled $\psi_e$ and $\psi_n$ are written as product combinations of ‘basis wave functions’ given in Equation (20):

$$|\psi_n\rangle = |I, m_i\rangle = \left| \frac{5}{2}, m_i \right\rangle, m_i = -\frac{5}{2}, -\frac{3}{2}, -\frac{1}{2}, \frac{1}{2}, \frac{3}{2}, \frac{5}{2}$$

$$|\psi_e\rangle = |S, m_s\rangle = \left| \frac{1}{2}, m_i \right\rangle, m_i = -\frac{1}{2}, \frac{1}{2}$$

Equation (20)

The basis wave functions are quantised along a nominated $z$ direction
As a consequence of these basis eigenstates, the combined Hamiltonian will always be represented as a square matrix, in this case it is a $2^6 6^4$ by $2^6 6^4$, 3888 by 3888 square matrix.

Equation (21) shows the general form of a basis wave-function of the combined “Mn$_4$+e” spin system:

\[
\psi_e, \psi_1, \psi_2, \psi_3, \psi_4 = |S,m_s|I_1,m_1|I_2,m_2|I_3,m_3|I_4,m_4\rangle \quad \text{Equation (21)}
\]

These vectors are orthonormal vectors, which means they follow the rule below (Equation (22), $m,n$ denoting different specific basis vectors)):

\[
\langle m | m \rangle = 1
\]
\[
\langle m | n \rangle = 0 \quad \text{Equation (22)}
\]

In order to solve for the eigenvalues, there are two methods that we have adopted, the second-order perturbation method and the matrix-diagonalisation method [30]. The former is a largely analytical quantum mechanical approach that provides only an approximate answer, but for most cases it is accurate enough, and it requires much less computing resources comparing to the latter method. That is a full quantum mechanical calculation, employing matrix algebra-executed numerically in almost all cases. It provides exact answers for the eigenvalues (to the limits of computational precision), but is very time consuming. In cases of our interest here, it may require weeks, or even up to months of super-computer time to calculate a single full simulation, so it is only feasible to use it as the last check after the perturbation calculations. Before we examine the two methods in detail, it is useful to first perform a series of simplified calculations with the Hamiltonian to have a more detailed understanding.
2.3.3 Examples of the terms in the Hamiltonian

Here we should first write the complete Hamiltonian for the “Mn4+e” spin system in Equation (16). The superscript, following Equation (19), indicates each specific Mn.

\[
H = H_{NZ}^{e} H_{NZ}^{Mn1} H_{NZ}^{Mn2} H_{NZ}^{Mn3} H_{NZ}^{Mn4} + H_{EZ}^{e} H_{EZ}^{Mn1} H_{EZ}^{Mn2} H_{EZ}^{Mn3} H_{EZ}^{Mn4}
\]

Equation (23)

- **Electron Zeeman**

The Electron Zeeman and the Nuclear Zeeman effects are very similar in terms of calculation, so we will use the Electron Zeeman as the example. We start from Equation (7), and define the direction of the magnetic field in the laboratory frame as the z-direction, i.e. \((H_Z \neq 0, H_Y = H_X = 0)\). Then we have Equation (24):

\[
H_{EZ} = \beta B_z \begin{bmatrix}
0 & 0 & 1 \\
g_{xx} & g_{yx} & g_{zx} \\
g_{zx} & g_{yy} & g_{zy} \\
g_{zy} & g_{yx} & g_{zz}
\end{bmatrix} \begin{bmatrix}
S_x \\
S_y \\
S_z
\end{bmatrix}
\]

Equation (24)

Then we can calculate this Hamiltonian to obtain Equation (25):

\[
H_{EZ} = g_{xx} \beta B_z S_x + g_{yx} \beta B_z S_y + g_{zx} \beta B_z S_z
\]

Equation (25)
The $S_x$ and $S_y$ terms in the equation can be represented in terms of the raising and lowering operators as in Equation (26):

$$S_x = \frac{1}{2}(S_+ + S_-)$$

$$S_y = \frac{1}{2i}(S_+ - S_-)$$

Equation (26)

Such that:

$$H_{Ez} = \frac{\beta B_z}{2} [S_+ (g_{s+} - ig_{s-}) - S_- (g_{s+} - ig_{s-})] + g_s \beta B_z S_z$$

Equation (27)

Consider initially the electron only wave function $\lvert s, m_s \rangle$, where $s$ is the total spin quantum number and $m_s$ is the magnetic quantum number. We have the Hamiltonian of the Electron Zeeman applying on one of the matrix terms, for the simplest case, we would have Equation (28):

$$H_{Ez} \lvert \frac{1}{2}, -\frac{1}{2} \rangle = \frac{\hbar \beta B_z}{2} \left[ S_+ (g_{s+} - ig_{s-}) - S_- (g_{s+} - ig_{s-}) \right] + 2h g_s \beta B_z S_z \left[ \frac{1}{2}, -\frac{1}{2} \right]$$

Equation (28)

For the electron wave function with $s=1/2$, $m_s=-1/2$, we know the raising and lowering operators work as below:

$$S_\pm \lvert s, m_s \rangle = \hbar \sqrt{s(s+1) - m_s(m_s + 1)} \lvert s, m_s \pm 1 \rangle$$

$$S_z \lvert s, m_s \rangle = h m_s \lvert s, m_s \rangle$$

Equation (29)
Together we have Equation (30), for a matrix element:

\[
\langle \frac{1}{2}, -\frac{1}{2} | H_{EZ} | \frac{1}{2}, -\frac{1}{2} \rangle = h\beta B_z \left( \langle \frac{1}{2}, -\frac{1}{2} | g_{zz} - ig_{zy} \rangle | \frac{1}{2}, \frac{1}{2} \rangle - \langle \frac{1}{2}, -\frac{1}{2} | g_{xx} - ig_{xy} \rangle | \frac{1}{2}, -\frac{3}{2} \rangle + \langle \frac{1}{2}, -\frac{1}{2} | 2g_{zz} | \frac{1}{2}, -\frac{1}{2} \rangle \right)
\]

Equation (30)

Note that the 1\textsuperscript{st} term in the answer becomes 0, because of the orthogonality of \(| \frac{1}{2}, -\frac{1}{2} \rangle\) and \(| \frac{1}{2}, -\frac{1}{2} \rangle\), and the 2\textsuperscript{nd} term annihilates as there is no electron spin -3/2 state that exists here; so only the 3\textsuperscript{rd} term is left, and we have Equation (31):

\[
\langle \frac{1}{2}, -\frac{1}{2} | H_{EZ} | \frac{1}{2}, -\frac{1}{2} \rangle = \frac{1}{4 \pi h} h\beta B_z g_{zz}
\]

Equation (31)

The Nuclear Zeeman calculation is very similar to the Electron Zeeman, hence it is not presented here in detail.

- **Hyperfine**

From Equation (12), the Hamiltonian of the hyperfine interaction is expressed as:

\[
H_{HF} = H_{HF}^{\text{iso}} + H_{HF}^{\text{aniso}} = \hbar a S \cdot I + \hbar S \cdot \vec{A} \cdot I
\]

Equation (32)

We first express the anisotropic term as Equation (33) (frequency units):
\[
H_{HF}^{aniso} = \begin{bmatrix}
S_x & S_y & S_z
\end{bmatrix}
\begin{bmatrix}
A_{xx} & A_{yx} & A_{zx} & I_x \\
A_{xy} & A_{yy} & A_{zy} & I_y \\
A_{xz} & A_{yz} & A_{zz} & I_z
\end{bmatrix}
\]

Equation (33)

Then we have:

\[
H_{HF}^{aniso} = S_x[A_{xx}I_x + A_{yx}I_y + A_{zx}I_z] + S_y[A_{xy}I_x + A_{yy}I_y + A_{zy}I_z] + S_z[A_{xz}I_x + A_{yz}I_y + A_{zz}I_z]
\]

Equation (34)

The isotropic part is then relatively simple, shown in Equation (34):

\[
H_{HF}^{iso} = q[S_xI_x + S_yI_y + S_zI_z]
\]

Equation (35)

- **Quadrupole**

For the quadrupole Hamiltonian term, we have

\[
H_Q = \hbar \mathbf{I} \cdot \mathbf{\tilde{Q}} \cdot \mathbf{I}
\]

Then we have Equation (35) (in frequency units):

\[
H_Q = [I_x \ I_y \ I_z]
\begin{bmatrix}
Q_{xx} & Q_{yx} & Q_{zx} \\
Q_{xy} & Q_{yy} & Q_{zy} \\
Q_{xz} & Q_{yz} & Q_{zz}
\end{bmatrix}
\begin{bmatrix}
I_x \\
I_y \\
I_z
\end{bmatrix}
\]

Equation (36)

which expands as;
\[ H_0 = Q_{xx}I_x^2 + Q_{yy}I_y^2 + Q_{zz}I_z^2 + Q_{xy}(I_xI_y + I_yI_x) + Q_{xz}(I_xI_z + I_zI_x) + Q_{yz}(I_yI_z + I_zI_y) \]

Equation (37)

The methods used in calculating matrix elements for the hyperfine and quadrupole terms are similar to those of the Zeeman terms. Each component of the matrix element is obtained separately, and then combined. However, due to the large number of matrix elements (15,116,544 in total), the full matrix is not presented in this thesis.

2.3.4 The spin relaxation and \( T_1, T_2 \) and the spectra line shape

The Electron Zeeman case can be used again to simplify the problem here. As illustrated in Figure 2.2.1, the population difference between the two separated Electron Zeeman states is given by Boltzmann’s Law:

\[ \frac{N_\beta}{N_\alpha} = e^{\frac{(g\beta H_0)}{kT}} \]

Equation (38)

This states that as the strength of the external field increases, the population of the \( \uparrow \) state (lower energy) \( (S=-1/2) \) progressively increases above the \( \downarrow \) \( (S=1/2) \) state.

The separation in energy between the two spin states for the electron in the applied field results in a difference in population between the two states. This population difference determines the net absorption of applied microwave energy at the resonance condition. The absorption of microwaves leads to a reduction in the population difference between the states. This change in net electron magnetic polarisation must be lost to restore the initial
spin state population. There are two physical pathways by which the net electron spin magnetisation can decay: spin-lattice relaxation and spin-spin or transverse relaxation.

After applying the external magnetic field, the $\beta$ state ($m_s = -\frac{1}{2}$) is now more favoured, so there must be interactions between the electrons and the surroundings to change the orientation of the electrons at thermal equilibrium. The excess magnetic energy of the $\alpha$ state ($m_s = \frac{1}{2}$) is transferred to other degrees (thermal vibrational etc) of freedom. Such a process is called a spin lattice relaxation, which is a non-radiative energy transition.

The rate of change of the population of $\beta$ state can be shown to equal:

$$\frac{dN_{\beta}}{dt} = N_{\alpha}W_{\alpha\beta} - N_{\beta}W_{\beta\alpha}$$

Equation (39)

$W_{\alpha\beta}$ and $W_{\beta\alpha}$ are the transition probabilities between the two spin states. If we define the population difference $n = N_{\beta} - N_{\alpha}$, a function of $t$, and $n_0$ to be the population difference at a particular time, i.e. to define the origin of time (normally thermal equilibrium), then:

$$\frac{dn}{dt} = -\frac{(n - n_0)}{T_1}$$

$$T_1 = \frac{1}{(W_{\alpha\beta} + W_{\beta\alpha})}$$

Equation (40)

$T_1$ here has dimensions of time, and hence it is named the “spin lattice relaxation time”, a measurement of the energy transfer rate to other degrees of freedom.

Typically, absorption spectra in ESR show two basic line shape extremes: a Lorentzian or Gaussian line shape. The Lorentz line shape normally appears in spectra taken from solution,
which has little relevance to the current project. In solids, whose spectra are normally ‘inhomogeneously broadened’, the total line shape for a particular transition usually has the form of a Gaussian curve:

$$g(\omega) = \frac{T_2}{\sqrt{2\pi}} e^{-\frac{1}{2} \left( \frac{T_2}{T_2} (\omega - \omega_0)^2 \right)}$$

Equation (40)

The term $T_2$ is called the transverse (or spin spin) relaxation time, which arises in solids mostly from a spread of precise relative energies of the spin levels associated with a particular transition (due to local inhomogeneities unaveraged on the ESR time scale), but is also contributed to by $T_1$ like processes. Typically, $T_2 \ll T_1$ [23].

2.3.5 The rotation matrix and Euler angles

Typically, nature complicates a problem and the principal axes of all the tensors $\tilde{g}$, $\tilde{A}$, $\tilde{Q}$, do not align within a system. They are variably rotated within the molecular frame and the calculation must take this into account in the simulation.

The choice of a reference frame is then rather arbitrary when the system has little or no natural symmetry (as here). We may then artificially choose a reference frame, and every tensor would then have their orientation determined by rotation, using Euler angles, relative to this reference frame. In the present particular case, we choose the principal axes of the hyperfine tensor of Mn$_1$ to be the reference frame, and every other tensor is referred in its rotation relative to this Mn$_1$ frame. These rotations were originally extracted from computational DFT calculations, then subsequently adjusted empirically. The detailed procedure is explained in the method and result sections.
To mathematically represent the rotations relative to the reference frame, we must first introduce the rotational matrix [30].

The rotational matrix $R$ is a 3 by 3 matrix that defines the relative orientation between two orthogonal Cartesian coordinate systems, so-named $xyz$ and $XYZ$ for this exercise. It represents the relative orientation between $xyz$ and $XYZ$ using three successive rotations, $\alpha$, $\beta$, and $\gamma$, which are called the Euler angles. A pictorial illustration is given in Figure 2.2.3.

The three rotations are:

1. $\alpha$-rotation $xyz$ counter-clockwise around the $z$-axis to give $x^1y^1z^1$.
2. $\beta$-rotation $x^1y^1z^1$ counter-clockwise around the $y^1$-axis to give $x^2y^2z^2$.
3. $\gamma$-rotation $x^2y^2z^2$ counter-clockwise around the $z^2$-axis to give $XYZ$.

There are up to 12 different conventions of the rotational matrix and Euler angles, and this particular one is called the $z$-$y$-$z$ convention, which is widely used in EPR related work.

The complete rotational matrix is a combination of three separate rotations represented by matrices as Equation (41):

$$ R = R_z(\alpha) \cdot R_{z1}(\beta) \cdot R_{z2}(\gamma) $$

Equation (41)
When we express this in matrix format, we have Equations (38) and (39):

\[
R = \begin{pmatrix}
\cos(\alpha) & \sin(\alpha) & 0 \\
-sin(\alpha) & \cos(\alpha) & 0 \\
0 & 0 & 1 \\
\end{pmatrix}
\begin{pmatrix}
\cos(\beta) & 0 & -\sin(\beta) \\
0 & 1 & 0 \\
\sin(\beta) & 0 & \cos(\beta) \\
\end{pmatrix}
\begin{pmatrix}
\cos(\gamma) & \sin(\gamma) & 0 \\
-sin(\gamma) & \cos(\gamma) & 0 \\
0 & 0 & 1 \\
\end{pmatrix}
\]

Equation (42)

\[
= \begin{pmatrix}
\cos(\alpha)\cos(\beta)\cos(\gamma) - \sin(\alpha)\sin(\gamma) & \sin(\alpha)\cos(\beta)\cos(\gamma) + \cos(\alpha)\sin(\gamma) & -\sin(\beta)\cos(\gamma) \\
-\cos(\alpha)\cos(\beta)\sin(\gamma) - \sin(\alpha)\cos(\gamma) & -\sin(\alpha)\cos(\beta)\sin(\gamma) + \cos(\alpha)\cos(\gamma) & \sin(\beta)\sin(\gamma) \\
\cos(\alpha)\sin(\beta) & \sin(\alpha)\sin(\beta) & \cos(\beta) \\
\end{pmatrix}
\]

Equation (43)

Figure 2.3 The graphical illustration of Euler angles.
(Image source: http://easyspin.org/easyspin/documentation/img/eulerangles.png)

In the simulations performed here, tensor rotations are represented using \(\alpha\), \(\beta\), and \(\gamma\) angles.
2.3.6 Powder Pattern

All the EPR spectra in this project were taken under cryogenic temperature conditions (typically 5-10K). Then the PSII sample material becomes a glass like solution. The PSII cores are randomly orientated centres, forming a powder pattern. Since each orientation has equal probability, the simulation needs to be solved for all possible field directions. This is achieved also by using the rotational matrix procedure introduced earlier.
CHAPTER III ELECTRON NUCLEAR DOUBLE RESONANCE
3.1.1. Introduction to ENDOR

ENDOR, or “electron nuclear double resonance”, was first introduced by Feher in 1956 [31a] b)) during solid state physics experiments, and was later expanded to measuring radicals in solutions by Hyde and Maki in 1964 [32]. ENDOR combined with EPR has been the most important tool for researchers working in photosynthesis [33], due to the fact that paramagnetic species appear very commonly in photosystems.

ENDOR is generally considered to be related to EPR, but it should not be confused with EPR. In fact it refers to a specific type of spectroscopy of magnetic resonance that is more similar to NMR. It concentrates on the study of the hyperfine couplings between an unpaired electron or electrons and a neighbouring nuclear, and detects transitions of $\Delta m_i = \pm 1$, as shown in Figure 3.1 (“red transitions”, this is a replica of Figure 2.2, shown here for convenience) [23].

There are two major branches of ENDOR, continuous wave (CW) ENDOR and pulsed ENDOR, which will both be introduced in this chapter.
Figure 3.1 The combined effects of the nuclear and electron Zeeman interactions, and the hyperfine coupling on the eigenstates and eigenenergies of the hydrogen atom spin system.
3.1.2. The spin Hamiltonian approach

Again, if we examine a simplified system of a single electron (S=1/2) and a single nucleus (I=1/2), such as a hydrogen atom (Figure 3.1), we have the Hamiltonian of the system shown in Equation (44):

\[ H = H_{N} + H_{E} + H_{HF} \]  

Equation (44)

We can then express the terms into Equation (45):

\[ H = -g_s \beta_B \cdot I + g_B \cdot \vec{g} \cdot \vec{S} + haS \cdot I + hS \cdot \vec{A} \cdot I \]  

Equation (45)

We can then simplify the matter further for the purpose of this exercise by dropping the anisotropic term of the hyperfine Hamiltonian, i.e. assuming the system is isotropic, which is often a good approximation and sensible in the case of a hydrogen atom:

\[ H = -g_s \beta_B \cdot I + g_B \cdot \vec{g} \cdot \vec{S} + haS \cdot I \]  

Equation (46)

The same idea follows for more complicated systems such as the manganese cluster in the OEC.
3.2 EXPERIMENT

3.2.1. Experimental techniques

Unlike other types of spectroscopic techniques, which use electromagnetic radiation from a particular range, ENDOR requires a combination of microwave (MW) and radiofrequency (RF) radiation excitations to conduct the experiment. This shows explicitly that it is a combination of EPR (MW radiation) and NMR (RF radiation) techniques.

Just as in a standard EPR experiment, the sample of interest would be placed in a primary magnetic field ($B_0$). Then secondary magnetic fields (from the MW) are applied to saturate a specific EPR transition, which would induce a near equal population between two spin states, e.g. $m_s = +1/2$ and $-1/2$, for a spin 1/2 system that we introduced earlier. Then an RF frequency would be applied to partially saturate the NMR transitions, which would also partly lower the saturation of the EPR transitions. Hence signals would be observed following the selection rules of NMR as shown in Figure 3.1, and hyperfine coupling constants could be extracted from the spectra, this would be more clearly seen once results are introduced in later chapters. The equations showing the saturation requirements are listed below:

\[
\gamma_e^2 B_1^2 T_e T_{2e} \geq 1
\]  
\[
\gamma_n^2 B_1^2 T_{1n} T_{2n} \geq 1
\]

Here the $\gamma$ are gyromagnetic ratios for electron and nuclear centres, the $B_1$ values are the relevant MW and RF radiation field intensities applied, and $T_1$ and $T_2$ are the relaxation times, as introduced before.
3.2.2. Continuous wave (CW)

There are various types of ENDOR experimental techniques in common use. One of the basic ENDOR techniques is the so-called continuous wave ENDOR.

Historically CW ENDOR was the first invented. Its RF radiation source is modulated, and the primary applied field $B_0$ is maintained constant, which is not the same as in an ordinary EPR field scanning experiment. Experimentally, one obtains initially the conventional first derivative spectra, and these observed EPR transitions are saturated by microwave irradiation, then desaturated by the applied RF radiation. This would be observed in the CW ENDOR experiment as changes in the EPR signal amplitude as a function of the frequency of the RF field, meaning that both microwave and radiofrequency fields are in resonance with the corresponding EPR and NMR transitions respectively, and the transitions of interest share a common energy level.

The ENDOR signal intensity, $E$, can be described by the following equation:

$$ E = \frac{1}{2(2 + b + b^{-1})} $$  \hspace{1cm} (45)

Here $b=W_n/W_e$, where $W_n$ is the rate of longitudinal spin relaxation of the nuclei, and $W_e$ is the corresponding value for the electrons [34].

This is a relatively simple and reliable technique, but in recent years, due to more advanced technological developments, other techniques such as pulsed experiments have been introduced to enhance the utility of the ENDOR spectroscopy. Hence the CW ENDOR has fallen somewhat out of use.
3.2.3. Pulse and Davies ENDOR

The nuclear coupling experiment that was conducted in this work for the OEC manganese cluster involved a more advanced and complicated technique known as Davies ENDOR.

Pulse manipulations of magnetisation are introduced in Davies ENDOR experiments, and the technique has several advantages compared to the traditional CW ENDOR. Pulsed ENDOR usually achieves an enhanced resolution and it is able to observe weakly coupled nuclei. It has been the most popular technique to be employed in EPR and ENDOR in recent years, due to its more advanced features.

Before conducting a pulsed ENDOR experiment, an electron spin echo (ESE) field sweep is performed to determine the field positions of EPR transitions of interest, to identify the primary field, $B_0$, values to be applied. The magnetic field value $B_0$ is then held constant, rather than scanning through a series of field positions in an ordinary CW EPR experiment. The ESE signal is created by the proper microwave pulse sequence, and a series of RF excitation sequences are then applied as pulses (rather than continuous waves in a standard NMR experiment) to sweep through the experimental field, in order to observe the “NMR-like” transitions as explained before. Pulse ENDOR signals are measured as the amplitude changes of the electron ESE signals, while scanning the radiofrequency.

There are two commonly used types of ENDOR sequences, Davies ENDOR and Mims ENDOR [35]. Davies ENDOR is used in our experiments, as the technique is most effective with nuclei of large hyperfine coupling constants, such as the manganese nuclei in the OEC.
Davies ENDOR starts with a preparation $\pi$ pulse, which inverts one EPR transition in the applied static $B_0$ field. This creates a “hole” in the EPR spectrum, whose width and depth are determined by the length of the applied pulse. Then follows a complete microwave inversion-recovery pulse sequence ($\pi-T-\pi/2-\tau-\pi-\tau$-echo) as illustrated in Figure 3.2. The $\pi$ pulse inverts the polarization, which is positive for a stable unpaired electron species in thermal equilibrium. Between the first and second microwave pulse trains there is a $T$ interval, during which time, an applied radiofrequency pulse changes the population of the nuclear sublevels associated with the main EPR transition, hence partially restoring the inverted polarization. Then the final microwave sequence determines the ESE signal intensity of this change, generating the ENDOR signal for the particular radio frequency applied. This becomes clearer once the experiments are introduced later.
4.1 SIMULATION METHODS

4.1.1 Introduction to simulating the multiline signal

As discussed above, the simulation involves solving the Hamiltonian matrices and obtaining their eigenvalues. Many research groups have presented simulations of the multiline (ML) signal in the past, generally providing reasonable overall fits to the data at modest resolution. However, no group so far has successfully explained the “super-hyperfine-structure” seen reproducibly within the spectra, i.e., the finer splitting within the main peaks of the multiline signal (e.g. see Figures 6.1 and 6.2 below). Providing a complete solution to this question has been the primary aim of this project, with a generally successful outcome. This will be developed in later chapters.

The S2 multiline signal contains 18-20 resolved hyperfine peaks, centred close to $g = 2.00$, with little $g$ anisotropy and is at least 180 mT (i.e. ~ 5,000 MHz) wide at X Band frequency (one peak is typically obscured by the strong central radical Yd signal near $g = 2.00$ [3, 11]). It may be conveniently generated in high yield by continuous illumination at low (~ 200K) temperature with visible (typically green) light [12]. It has long been recognized that the signal bears a superficial resemblance to the ‘classic’ 16 line hyperfine EPR signals seen in anti-ferromagnetically coupled Mn$^{III-IV}$ oxo bridged dimers at liquid He temperatures (e.g. [13]). The latter signals are generally < 130 mT in total width, significantly narrower than the S2 ML signal with fewer resolved lines. However, the multiline is itself narrower, with fewer lines, than the EPR pattern of the only example (known to the author) of an authentic Mn$^{III}$ Mn$^{IV}_3$ net spin 1/2 Mn tetramer (~26 lines, ~195 mT [14]).
Historically, two approaches have been taken to modelling the multilines, i.e., the magnetic coupling and Mn hyperfine interactions of the $S_2$ OEC cluster; Mn tetramer or effective dimer (or ‘near dimer’) models. A number of broadly similar tetramer systems have been proposed, with an Mn$^{III}$Mn$^{IV}_3$ oxidation pattern. Work up to 2012 is reviewed in [36]. Two dimer or near dimer models have been suggested [37]. These require a means to increase the width expected from conventional Mn$^{III}$ and Mn$^{IV}$ hyperfine parameters, to give a pattern $> 170$ mT wide. A large, rhombic anisotropic Mn$^{III}$ hyperfine tensor, with substantial quadrupole, was assumed in [37a]. In [37b] all tensors were assumed isotropic, with two contributing $\sim 2/3$ of the pattern width, i.e. dimer like, and one other being small. The PS II structures show all OEC Mn ions are close enough (with oxo, carboxylato bridging) to significantly exchange couple (confirmed by computational chemical studies eg see [38]). Thus for dimer like models to be applicable, two Mn (in states III and IV) must combine with net spin 1/2 and large effective hyperfine couplings, while two (both of the same oxidation state, III or IV) must couple to be largely ‘silent’, i.e. net anti-ferromagnetic, spin $\sim 0$. The net coupling between the two Mn pairs must be relatively weak. This is possible for ‘linear topology’ Mn systems [39] and perhaps the OEC Mn cluster geometry also [38]. Both require some small, or near cancelling exchange couplings. Generally, tetramer models predict effective hyperfine contributions for all Mn to be near isolated ion values or greater (for one Mn III). Dimer like models require one hyperfine interaction in the isolated ion range ($\sim 200 - 300$ MHz), one about twice this, as well as interactions below $\sim 100$ MHz, to give a ML pattern of $\sim 180$ mT width.
4.1.2 Introduction to simulating the S₂ ENDOR signal

In principle $^{55}$Mn ENDOR studies on the ML signal should resolve this matter, as ENDOR reveals the individual coupling frequencies directly. Several groups have now reported such studies, at X band and Q band on PS II preparations from several sources (plants, cyanobacteria) and with various levels of cryoprotectant and related small molecule species (e.g., MeOH) present, which are known to have subtle effects on the ML signals seen at low temperature. Such measurements were first performed by Britt et al. [29c, 33d], with subsequent studies mainly by Lubitz, Bittl and co-workers [33]. All of these report essentially the same general results - the only Mn derived resonances seen are from hyperfine couplings in the range ~ 170-340 MHz, observed as a broad, partially resolved envelope of somewhat variable shape, but typically peaking in the range ~ 240-280 MHz (i.e., at 120-140 MHz in the ENDOR spectrum). No resonances from couplings above 400 MHz or below ~ 160 MHz are reliably seen.

Although the technique used, Davies pulsed ENDOR, does not permit a quantitation of the number of Mn centers contributing to the total ENDOR spectrum (i.e., absolute signal intensities), the observation of some partially resolved structure in the envelope and absence of resonances outside the 170-340 MHz range, has led to the conclusion that three near isotropic Mn hyperfine couplings of broadly similar magnitude (~230 MHz) and one axial anisotropic coupling with perpendicular component ~ 350 MHz are present. The latter is assigned to a single Mn$^{\text{III}}$ and the former to three Mn$^{\text{IV}}$ ions in high oxidation state models.

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1 There is one report in the public domain from the Lubitz, Bittl group (then at the Technical University, Berlin) of $^{55}$Mn ENDOR couplings in the range ~ 550-600 MHz, reproducibly observed on the ML signal from spinach PS II membrane particles: Kammel, M. (2003) ‘Cofactors on the donor side of Photosystem II investigated with EPR techniques’. PhD Thesis: Faculty of Mathematics and Science, Technical University, Berlin.
All ENDOR techniques are sensitive to nuclear relaxation rates to some degree, CW ENDOR is fundamentally dependent on these (and electron relaxation rates), but even the pulsed techniques, such as Davies ENDOR, require that the nuclear $T_1T_2$ product be sufficiently long so that nuclear magnetization can be coherently rotated (or saturated) by the radio frequency pulses used in detection (eg. [39]). In paramagnetic systems, particularly at cryogenic temperatures, the nuclear relaxation rates will depend strongly on the electron spin relaxation rate (particularly $T_1$) and the coupling of this to the nucleus, through hyperfine fields and their fluctuation by matrix phonon etc. processes. All these effects generally have high intrinsic temperature dependences near 0K, dropping rapidly with decreasing temperature. This raises the possibility that nuclear relaxation may have hindered previous detection of some ENDOR transitions in the ML spin center (particularly those from Mn$^{III}$ ions), as all $^{55}$Mn ENDOR spectroscopy prior to completion of this work on the ML has been performed at liquid He (4.2 K) temperature or above. In this work (see Results) the first such studies at temperatures down to 2.5 K are described, using highly active core complex PS II samples from spinach (see Methods), together with complementary CW studies at X and Q band frequencies. $^{55}$Mn ENDOR hyperfine couplings ranging from ~ 50 to ~ 670 MHz are now seen on the $S_2$ state ML signal.
4.1.3 The matrix-diagonalisation method

Two types of mathematical tools are widely used to simulate EPR and related experiments: the matrix diagonalization method solves the complete Hamiltonian, and it is also the more “accurate and complete” solution, but once the system of interest becomes large in size and requires a higher degree of computational power, the matrix method may be not readily accessible due to hardware limitations; then the 2\text{nd} order perturbation method is generally used, which is an approximate solution but requires less computing power [40]. Both methods are used in our experiments depending on situation: the effective 3-Mn system can use the diagonalisation method approach; but the 4-Mn system would use the 2\text{nd} order perturbation theory due to the large computing power required for the calculation.

The eigenvalues of a matrix $A$ can be obtained through solving the characteristic equation of that matrix $A$, shown in Equation (46):

$$\det(A - \lambda I) = 0$$  

Equation (46)

Here $A$ is an $n$ by $n$ square matrix, $I$ is a $n$ by $n$ identity matrix, and $\lambda$ is the eigenvalue of the matrix, corresponding to one of the $n$ number of roots of the matrix $A$ [28]. Here the eigenvalues would be the corresponding eigenenergy levels for EPR transitions of interest.

The construction of the matrix has been explained in earlier chapters (Chapter II). Due to the size of the matrix, which contains hundreds of thousands of elements, it will not be listed here. The reader should refer to Easyspin for a more detailed explanation of its methodology [51].
4.1.4 The 2nd order perturbation method

A second approach to solving this problem of determining the eigenvalues/vectors is the time-independent (steady state) perturbation method, with 2nd order correction. The perturbation method assumes the total Hamiltonian, $H$, may be written as Equation (47):

$$H = H_0 + H_p$$  \hspace{1cm} \text{Equation (47)}

The perturbation method is an approximate calculation of the actual eigenenergies of the relevant eigenstates. It works well for electron net spin $\frac{1}{2}$ systems, like the OEC, with many nuclear hyperfine levels, whose spacings at X band or above are small compared the electron Zeeman energies. Therefore, we are able to apply the perturbation method with 2nd order correction, and obtain eigenenergies with good approximation.

In Equation (47), $H$ is the total Hamiltonian, which is split into two time-independent parts: $H_0$ is the Hamiltonian of the unperturbed system and contains essentially all the terms that are diagonal in the energy matrix. $H_p$ is called the perturbation (ie. non diagonal terms) and affects the eigenstates and EPR spectrum on a small (but important) scale. We can understand the perturbation as placing the system of interest in a weak ‘energy field’ (electric, magnetic, molecular etc.). It can be written in the form of Equation (48):

$$H_p = eW$$  \hspace{1cm} \text{Equation (48)}

Here, $W$ is an operator and $e$ is a dimensionless real parameter that is much smaller than 1. Then we have Equation (49) and (50):

$$H\psi = (H_0 + H_p)\psi$$  \hspace{1cm} \text{Equation (49)}
\[(H_p = eW) \psi = E \psi \]  

Equation (50)

A formal solution of \( E \) in Equation (50) is obtained as an infinite series of terms involving increasing powers of the parameter \( e \). Convergence of the method is assured if \( e \) is sufficiently small. In 2\textsuperscript{nd} order perturbation theory, terms contributing to \( E \) in \( e^3 \) or higher powers of the parameter are ignored. This is normally adequate for X Band simulations involving electron and nuclear Zeeman and nuclear hyperfine terms, but is inadequate to treat nuclear quadrupole effects [40], which generally require full matrix diagonalisation.
4.2 X-BAND CW EPR SIGNALS OF PSII

4.2.1 EPR Signals of the S-states

PSII samples may be selectively advanced from the S\textsubscript{1} state to other S states using single turnover laser pulses. Each of the S states has been successfully isolated using PS II membrane samples, however, obtaining a high population of a given state, not contaminated by other states, is challenging.

Each S state has characteristic EPR signals, shown in Figure 4.1. Among all S-states, the spin 1/2 signal from S\textsubscript{2} has been studied most extensively. This signal – the “multiline”, named as it has many characteristic Mn nuclear hyperfine lines, is easily formed and should, in principle, provide the most readily interpretable spectroscopic information.

4.2.2 The multiline (ML) signal

The multiline signal was first reported in 1981 by Dismukes et al. [12], when a series of flashes (0-6) were given to a PSII thylakoid sample. This “multiline” appeared on the first flash. The signal was predicted to arise from a strongly exchange coupled mixed valence Mn dimeric complex, with a single net unpaired spin and complex Mn nuclear hyperfine interactions, because of its similarity to mixed valence Mn model compounds already known [35].

![Figure 4.2](image-url) (left) the first multiline signal observed; (right) the Mn dimeric complex first studied [12]
The multiline signal can be seen in both PSII membrane and core samples, from both higher plants and cyanobacteria. Sample preparation and related details are explained in Chapter V, as they relate to the aims in this project. An advanced protocol was developed at an early stage of this project.

Cyanobacterial PS II samples, such as from thermophilic species, have been used extensively for EPR studies due to their superior stability. However, they do not fully replicate some details seen in higher plant PSII, which is the main interest here. Thus, in order to achieve the experimental aims here, an alternative illumination method must be developed to obtain close to 100% turnover of the PSII samples, especially the cores. This part forms the initial stage of the project and details are given below (Chapter V).
5.1 PHOTOSYSTEM II SAMPLE

5.1.1. Introduction to PSII samples

In order to study the S states, particularly the S2 state multiline, high quality PS II samples are demanded. The detergent solubilised PS II membrane preparation, which is essentially a sub-fraction of intact chloroplast membranes, is very stable [42]. However, due to the high number of chlorophylls per reaction centre (~200 chl. per RC), in comparison with the ~35 chlorophylls per reaction centre in the PS II core samples, the PS II membrane sample has far fewer reaction centers than cores for the same concentration of chlorophylls. The core sample is the smallest unit that retains the ability to generate oxygen. It consists of the D1 and D2 reaction center protein subunits, CP43, CP47 antenna proteins universally. It also contains (in plants) various other surface associated polypeptides (33kDa, 23kDa, 16kDa), depending on preparation method [43].

![Figure 5.1 A high plant PSII core sample](image)
The laser pulse technique has been used to advance the OEC into each S state, in which a single flash takes the system from $S_1$ to $S_2$, with appearance of the multiline signal [12, 44]. As note above, recent studies by Thapper et al. showed a 40-50% turnover of the OEC ($S_1$-$S_2$) using membrane samples with a single flash; and a 10-15% turnover using PS II core samples (as employed by them); both measurements were performed at 20°C, with 6ns, 5Hz pulses [45].

5.1.2. PSII samples preparation and illumination techniques

Spinach PSII core preparation

PSII core samples were prepared from market spinach according to the method described by Smith et al. [43] Typical concentrations used were 0.9mg/mL-2.0mg/mL chlorophyll, which corresponds to 30-70 µM reaction centres. The samples were stored in a system buffer consisting of 0.4 M sucrose, 20 mM MgCl₂, 10 mM MgSO₄, 5 mM CaCl₂, 0.3 mg/mL DDM (dodecyl-maltoside), and 20 mM BIS-TRIS, pH 6.5 (HCl). PpBQ (phenyl-p-benzoquinone) electron acceptor was added to each sample, with a series of selected concentrations between 0-90 molecules per reaction centre.

Continuous and flash illumination

Before the addition of PpBQ, the artificial electron acceptor replacing Q₀, all samples were first continuously illuminated for 12sec, at 235K, with a green filtered light source (250W) giving single turnover. The samples were then dark adapted in ice water for at least 10mins, which should give a sample of ~ 100% $S_1$ state. Then the continuous illuminations were repeated and samples frozen to 77K (liquid N₂). The individual multiline intensities formed in this way were used as references of 100% $S_2$ turnover. Various samples were prepared for
different concentrations of PpBQ (results are shown below, Section 5.2.4) to compare the effects of artificial electron acceptor levels. After the addition of PpBQ each sample was given one flash using a 200mJ per pulse YAG laser, in a N₂ gas flow cryostat, between 273-291K, then stored in at 77K before EPR measurements. Properties regarding samples prepared, please refer to the published work of Smith et al. [43].
5.2 100% TURNOVER OF PSII CORE SAMPLE

5.2.1. Signal II₅ test

As illustrated earlier, the concentration of the sample is crucial in maximising signal to noise ratio, hence obtaining a reaction centre concentration as high as possible is a key issue for this project. The tyrosine₀ (Y₀⁺) signal was integrated to calculate an absolute stoichiometry of chlorophylls per reaction centre, which was estimated to be ~35 chlorophylls per reaction centre. This is essentially the theoretical stoichiometry and means the core samples used retained ~ 100% intact structure. Figure 5.2 provides an EPR signal of a typical tyrosine₀ (Signal II₅). Please refer to part 5.1 for sample preparations and properties.
5.2.2. Mn₄-S₂ Multiline Signal (ML)

Figure 5.3 100% multiline generated using a single flash

Figure 5.3 shows an example of a Mn₄-S₂ multiline EPR signal, generated in core complexes. In our experiments, a complete S₁ to S₂ turnover of the spinach PSII core samples was achieved using a single flash, at 270K, with ~20 PpBQ per reaction centre, and a minimum transfer delay between the flash illumination and the 77K frozen storage.

The reasons we are able to achieve a single flash induced 100% multiline turnover using PS II core samples, while other groups observe less than 20% turnover is addressed in detail below. However in summary, it was found that three factors affect the experimental ML yield:

1. The temperature of the illumination.
2. The concentration of the artificial electron acceptor.
3. The freezing technique.
5.2.3. General kinetics of the sample

There are some facts already known from previous studies regarding the kinetics of processes, in PS II, relevant to the present work.

Even though $S_0$ is the most reduced state, it is not the most thermodynamically stable state. This state is $S_1$. In a dark relaxed sample following multiple turnovers, the ratio between $S_0$ and $S_1$ is about 1:3. $S_2$, $S_3$ always spontaneously reduce to $S_1$, and $S_4$ is an intermediate transition state, that is not possible to isolate [46].

Before each flash experiment, the sample, without PpBQ, was continuously illuminated, then dark adapted (annealed) for at least 10 mins. Because only one $S$ state advancement could occur, a mixture of $S_1$ and $S_2$ states was present, as endogenous QA is the only electron acceptor present able to store an electron. After some back reaction cycling during illumination, a mixture of $S_1$ and $S_2$ is present. Then after the dark adaption step, all reaction centres were in the $S_1$ state. This ensures that the sample was completely in the $S_1$ state before the single flash that generated 100% $S_2$ [75].
5.2.4. Illumination technique

The flash-freezing apparatus is shown in Figure 5.4. The sample tube was placed in a N₂ flow cryostat with a constant temperature, which was controlled by pre-cooling the N₂ stream in a set temperature water bath. The emergent laser beam was expanded using a cylindrical biconcave lens to a long eclipse, which matched the sample length as closely as possible. After the sample is illuminated, there is a short time delay between the tube being taken out and then placed in the 77K environment. This delay proved to be crucial in understanding the kinetics of the Q₄S₁ – Q₄⁻S₂ reaction, which were studied in detail [75].

![Figure 5.4 the freezing apparatus](image-url)
Figure 5.5 the temperature dependence of the illumination, Peak 5 is an indicator of the intensity of the multiline signal, hence the percentage of turnover. The x-axis has the units of mT.

As shown in Figure 5.5, the percentage turnover has a very strong dependence on the temperature of illumination. Here we show results from 15°C, 9 °C, and -3°C illuminations. Peak 5 of the multiline is used as an indicator of turnover percentage, compared with a complete, continuous illumination, taken as 100%. The -3°C illumination has the strongest Peak 5, equivalent to the reference value, hence a 100% turnover is achieved with a -3°C illumination.
Figure 5.6 The Arrhenius Plot of back reaction (ML decay) kinetics (derived from Figure 5.5)

<table>
<thead>
<tr>
<th>Temp C</th>
<th>$t_{1/2}$ seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>6.2</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>18</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Table 5.1 on the left shows a detailed kinetic analysis of the half-life of the $S_2$ state at various illumination temperatures (see Fig 5.6 above).

The ML signal decay was determined by dark incubation of $S_2$ advanced samples, for increasing times at the nominated temperature, then freeze trapping and measuring the remaining signal intensity as in Fig 5.5, and the ML signal decay rates were found to be first order within the resolution of the data. It is clear that the lower the temperature, the slower the multiline decay. This is the reason why we could obtain a 100% turnover at -3°C (270K). With a 20°C illumination, the half-life of the multiline is <2 s, which was not sufficient to transfer the sample to stable low temperature storage. Temperatures lower than -3°C would theoretically better preserve the multiline, however, due to the aqueous nature of the PSII core sample, the solution freezes significantly below -3°C, which would in fact reduce the turnover efficiency of the sample. Hence -3°C is an optimum
condition. The sample does not freeze at 0°C because the various additives such as Mg\(^{2+}\)/Ca\(^{2+}\) salts and sucrose, lower the freezing point of the sample.

Figure 5.6 gives an analysis of the decay rate using the Arrhenius Equation:

\[
k = Ae^{-\frac{E_a}{RT}}
\]

\[
\frac{d \ln k}{d \left(\frac{1}{T}\right)} = -\frac{E_a}{R}
\]

Equation (51)

The plot is \(\ln(k)\) against \(1/T\), where \(k\) is the rate constant (here first order) and the absolute temperature is \(T\). \(A\) is the pre-exponent factor, \(E_a\) is the activation energy and \(R\) is the universal gas constant. Here the activation energy for multiline decay in our PSII core sample is \(E_a \approx 0.55\) eV, compared to \(E_a \approx 0.5\) - 0.7 eV in membrane samples [47]. The reaction occurring here is \(Q_A S_2 \to Q_A S_1\).
5.2.4. The artificial electron acceptor PpBQ

The “acceptor pathway” has been described in earlier chapters and the mobile two-electron carrier acts as the final stage of the electron transfer in PSII, where the Q₈ in the binding pocket is reduced to Q₈²⁻, taking two protons from the surrounding medium to become the quinol, Q₈H₂, before leaving the pocket [48]. However, almost every PS II core sample preparation method now known (especially higher plants), results in some loss of Q₈, hence only a single electron can be accepted by Qₐ to form Qₐ⁻ in these cases [49]. Without Q₈H₂ to remove electrons from the system, Qₐ⁻ back reacts thus reducing the turnover percentage.

To counter this problem, an artificial electron acceptor must be introduced to replace the function of Q₈. Phenyl-p-benzoquinone, or PpBQ, was chosen after a series of tests on various similar molecules found that PpBQ was the most efficient. In the native membrane there are about 7~8 Q₈ molecules/PS II [50]. However the requirement in the solubilized system may well be different and the concentration of PpBQ, which would provide the best turnover efficiency, must be empirically determined.

![Chemical structures](image)

*Figure 5.7 (from left to right) PpBQ, ubiquinone in the bacteria PSII, plastoquinone in the higher plant [47]*

Figure 5.8 shows the relationship between the PSII S₂ turnover extent in one flash, then freeze experiments, and the concentration of PpBQ added. This is defined as the number of
PpBQ molecules per reaction centre, at the various temperatures studied. The conclusion here is that at the critical illumination temperature (270K):

1. Too many PpBQ (~90 molecules per reaction centre) worsens the turnover efficiency.
2. No PpBQ added would also be an optimum reaction condition. That has an effective turnover efficiency (as only one electron is transferred between $S_1$-$S_2$, for which $Q_A$ is sufficient in principle to capture the transferred electron), provided no back reaction occurs. This is impossible in practice however, as the $Q_A$ captured electron can often backfire through acceptor pathway.
3. An effective addition of PpBQ is ~20 molecules per reaction centre is optimal, which was the actual additive concentration used during our experiments.

Figure 5.8 Effects of the PpBQ on PSII turnover extent, $R$ symbolises the concentration of PpBQ, measured in number of molecules per reaction centre.
5.2.6. The freezing technique

It is shown above that 270K provides the longest half-life time of the \( S_2 \) state, without causing other negative factors such as frozen solution. This long half-life is required to counter the sample transfer loss, i.e. the \( S_2 \rightarrow S_1 \) back reaction during sample transfer from the illumination apparatus into the 77K liquid \( N_2 \) storage, after which the sample is stable in the \( S_2 \) state until the measurement is taken. This time is very crucial as the most experienced hands take \( \sim 2s \) to execute the transfer. Within this 2s window, 90% of the \( S_2 \) could back react to \( S_1 \) if the illumination was conducted at room temperature (298K), hence the poor turnover efficiency seen by some other groups.

After the optimum illumination temperature has been determined, this 2s window can be easily accommodated with virtually no \( S_2 \) state loss, thus achieving a 99-100% turnover efficiency.
efficiency. This is evident from the kinetic analysis of the back reaction of $Q_A(S_2)$-$Q_A(S_1)$ shown in Figure 5.6.

Figure 5.10 Half-Life of the $Q_A(S_2)$-$Q_A(S_1)$ back reaction

As shown in the figure, any illumination temperature above 273K would result in significant loss after the 2s window.
5.2.7. Conclusion

It may be concluded that the spinach PSII core samples used in this work may be completely advanced into the $S_2$ state (100% of the multiline signal intensity) by single flash turnover, provided the following factors are controlled properly: the temperature at the time of illumination (should be 270K), the concentration of the PpBQ (~20 mole ratio on reaction centres), the time delay between the illumination and the 77K freezing-storage (~2s). This follows from the temperature analysis of multiline decay - the lower the temperature illuminated, the more tolerable the system is to the time delay.

From the temperature dependence of the $Q_{A}S_1\rightarrow Q_{A}S_2$ reaction kinetics, the activation energy was estimated to be ~0.55eV in the spinach PS II core samples, which is very similar to the value, 0.5-0.7eV in spinach membrane samples. Since every ~0.05eV change in activation energy results in ~10 fold difference in the reaction rate, even the modest energy difference above explains well why the back reaction of the $Q_{A}S_1\rightarrow Q_{A}S_2$ transition is some 20 times slower in membrane samples than in cores [47]. Although most detailed EPR studies of the multiline system in this work employed 200K green illumination turnover, for maximal signal intensity, the flash characterization of the core samples undertaken here was vital to demonstrate the full functionality of this system.
5.3 EXPERIMENTAL METHODS

5.3.1. X-Band CW EPR

The X-Band CW EPR measurements were performed at 6.5 K on a Bruker Elexsys E500 spectrometer using a Bruker SHQX resonant cavity, fitted with an ES900 helium flow cryostat with temperature controlled via an Oxford ITC4 controller. Spectrometer frequency; 9.377 GHz, microwave power; 20 mW and modulation amplitude; 10G. Field control by NMR Gauss meter.

5.3.2. Pulsed EPR and ENDOR Measurements

Pulsed EPR and ENDOR experiments were undertaken at the Center for Advanced Imaging, University of Queensland, using an ELEXSYS E580 Pulsed ENDOR spectrometer fitted with an Oxford CF935LT helium cryostat. Experiments below 4 K (down to ~ 2.5K) were achieved by He pumping (40 l/min Leybold roughing pump), with temperature controlled using an Oxford ITC 503 controller. The X,Q band spectrometer system employed an X-band ER4118XMD-4W1 ENDOR resonator, a DICE I ENDOR unit with a Amplifier Research 1-400 MHz radio frequency (RF) amplifier (150A400), a Bruker frequency counter and a Bruker ER036M teslameter for calibrating the microwave frequency and magnetic field, respectively.

Electron spin echo-detected (ESE) field-swept spectra were routinely measured using the pulse sequence: $\pi/2-\tau-\pi-\tau$-echo, where $\pi = 32$ ns and $\tau = 200$ ns to ensure measurement of either the $S_2$ ML state species or the background Mn $^{II}$ species. From these appropriate resonant field positions were identified and stochastically collected $^{55}$Mn-Davies ENDOR spectra were taken using the pulse sequence: $\pi-\pi$(RF)-$T-\pi/2-\tau-\tau$-echo over two overlapping
RF ranges (1-230 and 140-370 MHz), with timings: $\pi = 24$ ns, $\tau = 200$ ns, $\pi_{(RF)} = 6$ $\mu$s and a delay $T = 600$ ns.

The Bruker Biospin DICE I permits RF frequencies from 1-200 MHz and the $B_2$ output profile is not linear. The data collection range was extended to 400 MHz with the aid of a frequency doubler (15542, Mini-circuits). The frequency doubler slightly attenuates the signal, so the output from the frequency doubler was amplified using a BLAXXS300RS amplifier (from a Bruker NMR spectrometer) and then attenuated with a Tenuline coaxial attenuator (Model 8325, 30db) so as to not damage the 150W Amplifier Research RF amplifier.

5.3.3. X-Band CW-EPR and $^{55}$Mn-ENDOR Simulation

The $S_2$ state X-Band CW-EPR and $^{55}$Mn-ENDOR spectra were calculated interactively, to maintain consistency. Essentially the same Spin Hamiltonian was used for both simulations, which assumed that the effective spin of the ground state is $S=1/2$. For the multiline simulations, the three-Mn system was treated using the complete matrix diagonalisation method. For the four-Mn system the matrix approach was prohibitive computationally and this system was treated using second order perturbation theory. Spectral simulations were performed using the EASYSPIN package in MATLAB [51].

5.3.4. DFT Calculation details

The DFT calculations were performed using the ADF program, to obtain ‘single ion’ values of the EPR parameters suitable for comparison with the experimental data. Only Euler angles of the hyperfine tensors on each Mn were benchmarked for the simulations; these were used as inputs for subsequent refinement. All the structures calculated had been optimized.
without restrictions, using the Hybrid B3LYP functional and TZVP basis sets for all atoms. Geometries from the ‘Berlin’ (Type I) and ‘Hyogo’ (Type II) isolated clusters were used, as determined earlier by Petrie et al. [52].
6.1.1. The “narrow” and “broad” forms of the multiline

It has been known for some time that the $S_2$ state multiline signal as conventionally generated for EPR study is not always a homogeneous species ([37, 53], see also discussion in [11]). The origins of this effect, or effects, are yet to be fully defined, but PS II species (plant and cyanobacteria), cryoprotectant and the presence of small alcohols at non functionally inhibiting concentrations (typically $< 1 \text{ M}$) all influence the detailed, ‘super-hyperfine’ structure of the ML signal and number of resolved peaks. It has been shown by computational modelling of OEC geometries consistent with different crystal structure forms [54], that several near equi-energetic configurations of the Mn$_4$/Ca cluster are possible. These differ mainly in the relative orientation of the Mn$_3$/Ca quasi-cubane motif to the fourth (or ‘dangler’) Mn ion, which is ligated by only two protein-supplied groups (carboxylate side chains of D1 Asp 170 and Glu 333). This could underlay the observed lability of the site.

Phenomenologically, Pace et al. have identified two multiline signal forms, a so called ‘broad’ and ‘narrow’ form [11]. The ‘narrowness’ of the narrow form, routinely seen in the presence of MeOH, may however be no more than a consequence of poor resolution, or smearing of features near the ML signal edges.
Figure 6.1 Full width CW spectra of typical S2 Multiline forms of the ‘broad’ (red, PS II Cores) and ‘narrow’ (black, PS II Membranes, see text) types. Spectra are illuminated S2 – S1 differences. Central region (indicated) about g = 2 obscured by Tyrosine:subtraction artifact (see also Fig 2). Spectrometer conditions as in Methods and Table 1A,B.
Figure 6.2 Expanded detail of spectral region in 6.1 from 300-400 mT. Indicated are several examples of what appear as similarly patterned peak splittings that are consistently smaller in the narrow form, compared to the broad form. Spectrometer conditions as in Methods and Table 1A,B.

Fig 6.1 shows examples generally representative of both types of ML ($S_2 - S_1$ annealed, difference spectra), one from PS II core complexes and one from PS II membrane particles, both from higher plants (see Methods). A difference in signal to noise is apparent in these spectra, as the OEC concentration in the core complex samples is ~ seven times higher than in the membrane samples. Although formed in the presence of glycerol as cryoprotectant, the membrane spectrum is essentially the same as that seen for plant PSII in the presence of MeOH [4,15f]. The core complex spectrum is very similar in detailed shape to that of membrane PS II in the presence of EtOH, the most ‘pure’ broad form observed [4], although no mono-alcohol species are present in the PS II core samples. The spectra in Figure 6.1 are
virtually identical in main peak positions, but there is a clearly discernible difference in
detailed peak ‘fine structure’ in these high resolution CW spectra, particularly above \( g = 2 \)
(Figure 6.2). This is not an intrinsic line-width effect, as the sharpness of the reproducibly
resolved features in the narrow form is, if anything, greater than in the broad form. Rather
it appears that a repeating structure of order 80 MHz splitting (i.e. \( \sim 3 \) mT) in the broad form
is reduced to \( \sim 50 \) MHz (\( \sim 2 \) mT) in the narrow form. This structure must arise from an
anisotropic hyperfine interaction, as it is not seen fully on all main peaks (roughly every
second peak in the 300-400 mT region). A hyperfine interaction of this magnitude is
inconsistent with all high oxidation state paradigm tetramer modelling of the Mn cluster to
date.

6.1.2. X-band pulsed EPR studies, \( T_1 \) relaxation

All pulsed EPR studies were performed on detergent solubilised PS II core complexes from
spinach, developed for spectroscopic use (Methods). As noted earlier, this material has very
high catalytic turnover activity and low total chlorophyll per reaction centre (\( \sim 34 \) Chl\(_a\)), but
lacks the luminal associated PSB P,Q peptides, unlike PS II membrane particles or PS II from
thermophyllic bacteria (which possess analogous but different surface associated peptides).
As established in Chapter V, these PS II core complexes, typically at concentrations of 6-7
mg/ml Chl, permit efficient illumination induced turnover and have reaction centre
concentrations > 220 \( \mu \)M, which is beyond that normally attainable with PS II membrane
preparations.

The protocol consistently employed is to examine \( S_2 \) and \( S_1 \) state differences, where the \( S_1 \)
state is that formed after allowing the \( S_2 \) state to relax in the dark (at \( \sim 0 \) °C) for up to \( \sim 30 \)
mins. This is because other paramagnetic centres (cyt b$_{559}$, Tyrosine Y$_{d}$ radical, Q$_{A^{-}}$ center) form and are trapped by the 200 K green illumination used to maximally generate the S$_{2}$ ML signal, but these remain largely unaltered by the dark annealing that converts the OEC back to the S$_{1}$ state.

Fig 6.3 shows the echo detected field swept spectra of PS II complexes in the illuminated S$_{2}$ state, with spectra from partially and essentially fully relaxed (S$_{1}$ state) samples (all at same concentrations). The S$_{2}$ state spectrum shows six small sharp features from uncoupled Mn$^{II}$, which is typically found in these samples, at concentrations of ~2-3 % or less of reaction centres. It is likely of similar origin to the small concentration of uncoupled Mn$^{II}$ seen in Sr substituted cyanobacterial PS II [18e] and is unlikely to be non specifically associated, as it appears to ‘fade’ (somewhat variably) with annealing (possibly broaden), an effect also seen in Mn ENDOR spectroscopy (e.g. Fig 6.6 below). The nature of this uncoupled Mn$^{II}$ is not explored further here, but it may arise from perturbation of a small fraction of OEC sites, due to Ca/Sr replacement in one instance or loss of surface peptides in another. These sharp Mn$^{II}$ positions were avoided in ENDOR measurements (below). The S$_{2}$ $\rightarrow$ S$_{1}$ conversion kinetic itself was also found to be somewhat sample dependent, which might reflect the fact that the core complex S$_{2}$ states as prepared are probably still heterogeneous in some property, which reflects in the electron spin lattice rates (below, Figure 6.4).
Fig 6.3. Echo detected field swept spectra of PS II Cores. Freshly illuminated S$_2$ (black), partially annealed S$_1$ (~20 min dark, 0 °C, red) and fully annealed S$_1$ (>30 min dark, 0 °C, blue) All spectra aligned at common g =2.000 at frequency 9.634 GHz. Shown on spectrum of the S$_2$ state are the three field positions (g ~ 2.18 a, 2.05 b, 1.93 c) at which ENDOR measurements were made (see text). Oxidised cyt b$_{559}$ is present in these samples, (g$_x$ ~ 2.9 resonance around 235 mT), as is the radical signal from oxidized Tyr$_{60}$ near g = 2.00. The six small sharp features are from background Mn$^{II}$. 
Figure 6.4 Shows echo inversion recovery $T_1$ plots for the ML signal (measured near point b in Fig 6.3) at temperatures 2.5 K and 5.2 K. Plotted is $\ln$ [normalized amplitude] vs. time. At long times plots are linear (single exponential decay) but multi-exponential at short times. Fits (red) are to a mixed dipolar, with $F$ parameter = 0.60, 2.5 K and $F = 0.87$, 5.2 K (see the following section for the details of the dipolar model)
Multiline T₁ Relaxation:

- Dipolar relaxation model applied to PS II Cores

T₁ relaxation studies were performed on the ML state of the PS II cores used here. This was undertaken, in part, to explore why the ENDOR results obtained here differed from all earlier repeated ENDOR measurements on the ML S₂ state of PS II. The inversion recovery amplitude response, Aᵣ(t), for the ML spin lattice relaxation was fitted to a modified form of the dipolar interaction model, as discussed in [26]. This is of the form:

\[ \frac{A_r(t)}{A_r(0)} = F[DipolarModel] + (1-F)[e^{-k_{BACKGROUND}t}] \]  \hspace{1cm} \text{Equation (51)}

Aᵣ(0) is the initial amplitude of the inverted spin echo signal, (as conventionally defined- long time recovery limit is zero, see below). F is a fraction (0-1.00), which represents the fraction of centers experiencing non-exponential relaxation- interpreted as dipolar relaxation interaction with another, specific paramagnetic center within the PSII complex. k_{BACKGROUND} is a background, first order rate, which is presumed to operate in centres not experiencing the specific dipolar interaction and includes the effects of all other (including weak dipolar) relaxation mechanisms. Within the limits of the present analysis, these can be adequately represented by a single first order term.

The Dipolar Model, I(t), is of the form:

\[ I(t) = \int_0^{\pi/2} \sin \theta \left[ e^{-(k_{\text{scaler}} + k_{θ})t} \right] d\theta \]  \hspace{1cm} \text{Equation (52)}
and represents a powder average of the angular dependent dipolar relaxation interaction, given by $k_{1\theta}$, plus a first order scalar background interaction, $k_{1\text{scaler}}$. The $k_{1\text{scaler}}$ and $k_{\text{BACKGROUND}}$ terms are assumed equivalent, in a minimal model. [26]

The angular dependence of the $k_{1\theta}$ term is taken as;

$$k_{1\theta} = k_{1d}(1 - 3\cos^2 \theta)^2$$

Equation (53)

where $\theta$ is the angle between the vector joining the interacting spins and the external magnetic field. This assumes that the spin lattice relaxation enhancement by interaction with the specific fast relaxing centre is dominated by the leading B term in the dipolar interaction expression (see [26]). $k_{1d}$ is determined by the nature of the interacting spins and their separation. The inversion recovery data, as recorded, has an instrumental offset, which must be subtracted to obtain the true infinite time asymptotic response. This then allows determination of $A(0)$. The offset value was determined to give the best linearity of the long time log response of the data, after subtraction of the offset. The fractional uncertainty in the inferred parameter values for any individual fit is ~5-7%, for the three dipolar model terms, and ~1% for $A(0)$.

For the two ML echo decays in Fig 6.4, the parameters values are:

2.5 K: $F = 0.60, \quad k_{1d} = 1.0 \times 10^3 \, \text{s}^{-1}, \quad k_{\text{BACKGROUND}} = 39 \, \text{s}^{-1}$

5.2 K: $F = 0.87, \quad k_{1d} = 3.3 \times 10^3 \, \text{s}^{-1}, \quad k_{\text{BACKGROUND}} = 120 \, \text{s}^{-1}$
Researchers [55] have determined the electron spin lattice ($T_1$) rates of the MLS signal as a function of temperature and sample treatment ($\pm$ MeOH) for PSII from spinach membranes and thermophilic cyanobacteria. They find that MeOH has a much greater influence on the $T_1$ rates (presumed averages from bi-exponential fits [56], see also below) in plant, compared to cyanobacterial PS II. The temperature range examined was $\sim 6 - 4.2$ K. We have performed similar measurements on the ML signal from spinach cores, at 5.2 and 2.5 K. as seen above in Fig 6.4. The decay is exponential at both temperatures at long times, but faster and more complex at short times. A bi-exponential model does not fit the data closely, but it has previously been shown that a ‘mixed dipolar’ model as described above, well fits both the $S_0$ and $S_2$ state multiline ESEEM envelope decays (dynamic $T_2$ rates), as well as the $T_1$ rates [57a] for species near the OEC like Yd. [57b]. The dipolar model formally assumes the existence of a ‘fast paramagnetic relaxation centre’, interacting through space with the paramagnet of interest. If the fast species is effectively non Kramers (even spin) it might be difficult to detect in conventional CW EPR and this model cannot anyway be an exact description of the relaxation pathways within the coupled Mn cluster (the likely basis of its approximate validity however will become apparent below, when the nature of the spin coupling in the cluster is considered). Nonetheless for ready comparison with other data, an exponential average $T_1$ relaxation rate is determined for the core samples, defined by:

$$\frac{1}{T_{1AV}} = \int_0^\infty \text{Sig}(t)dt$$

Equation (54)
where $\mathrm{Sig}(t)$ is the (normalised to unity at $t = 0$) longitudinal echo amplitude decay. Also fast and slow component rate estimates for two exponential fits at both temperatures are given.

Figure 6.5 Comparison, as function of temperature, of mean electron $T_1$ relaxation rates of the ML centre in PSII samples from different species/solvent conditions etc., as indicated in figure insert (also see text). All data on Elongatus and spinach (membranes) taken from Cox et al [55]. Spinach core data from Fig 6.4. For the latter, the mean $T_1$ rate (see text) is plotted, as well as the fast and slow rates from two exponential fits to the data in Figure 6.4.

All rates are significantly slower than any rate observed by Cox et al. [55a] even at 2.5K [55b]. Figure 6.5 shows a comparison plot of $T_1$ temperature dependence data from those authors and the results in Figure 6.4. Although determinations at only two temperatures have been made, it is clear that the weak temperature dependence of the $T_1$ rate seen by Cox et al. for spinach PS II without MeOH is similar to that which is seen in both components for the spinach core complexes, although these are significantly slower in overall rate values. An
interesting phenomenology seems to emerge. The ML signal spin lattice relaxation has a relatively weak temperature dependence in the broad forms of plant PS II (core complexes and membranes without MeOH), while it has a strong dependence on temperature in the + MeOH narrow form, similar to that seen in cyanobacterial PS II (with or without MeOH). The ML signal $^{55}$Mn ENDOR spectra reported by Cox et al. show a significant influence of MeOH for spinach PS II, but not cyanobacterial PS II. Thus, whatever are the local changes wrought by MeOH in the OEC region of plant PS II, these seem to be largely ‘already in place’, within cyanobacterial PS II, at least by the criteria discussed above. This is of course totally consistent with the well-known functional insensitivity of PS II to MeOH, at the solvent concentration levels relevant here. No further examination of these matters is made here, but from Figure 6.5 the majority sample electron $T_1$ rates occurring in the present measurements at 2.5 K are at least 10 times or more slower than have likely applied in any $^{55}$Mn ENDOR measurements previously reported on the ML signal, even including the recent results from Krewald et al. at 2.5K [55b].

6.1.3. X-Band Davies ENDOR spectroscopy

$^{55}$Mn ENDOR studies on the PS II core complex material were performed mostly at 2.5 K, with some data acquired at 4.2 K, to compare with results published earlier by others. The frequency interval examined was 0-400 MHz, which should encompass the full range of Mn couplings likely to arise in dimer or tetramer OEC models. In a simple first order picture, frequencies corresponding to a Mn coupling of $A$ MHz, occur around $A/2$ MHz in the ENDOR spectrum, for this net electron spin $\frac{1}{2}$ system in which the hyperfine interactions strongly dominate over Zeeman and quadrupole terms.
Although simple in principle, there are a number of sample and instrumental dependent factors which may significantly influence (individually and by interaction) the outcome of a Davies \(^{55}\text{Mn}\) ENDOR experiment on a system as complex as the OEC. A number of these have been discussed in detail by Cox et al. [58] (see also [56]). However, briefly, three important ones, relevant here are:

1) The \(B_2\) profile across the RF radiation frequency range being sampled

2) The actual nuclear Hyperfine value itself (roughly its isotropic value, \(A_{\text{iso}}\))

3) The spin-lattice relaxation processes, in particular the Orbach Process [76]

4) Hyperfine orientation selection at the envelope position at which the pulsed experiment is performed.

Typically the RF frequency is randomly varied between shots in the data acquisition range, to prevent heating artifacts (Methods), but the \(B_2\) value at the specific frequency is not normally separately controllable, other than as an instrument dependent profile (a property of the RF synthesizer/amplifier), which may be attenuated by a preset value for the particular experiment. The \(B_2\) value at the RF frequency together with the hyperfine \(A_{\text{iso}}\) value determine the nuclear nutation ‘flip angle’ for a given RF pulse length (through hyperfine enhancement, see [56,58]) and this directly influences the intensity of the observed ENDOR response at the particular radio frequency. This is especially so when, as here, the hyperfine frequencies are typically much larger than the nuclear Zeeman frequencies (by factors of 10-100 at X Band).

Point 4 above is particularly important when the spin system contains large, anisotropic hyperfine components (as will be the case here). Then the narrow electron spin packets
excited at the particular magnetic field position of the ENDOR experiment do not sample equally the full powder pattern range of (some at least) hyperfine components, and particular molecular orientations are then selectively seen (or relatively enhanced) in the ENDOR response, even if, as for the ML center, the g tensor is near isotropic (below). This then influences the observed intensities of ENDOR lines in non obvious ways for complex, multinuclear systems (again as here).

To at least partially address points 1 and 2 above, the RF acquisition range was dived into two overlapping segments, 0 - 230 MHz (‘Low Freq.’) and 140 - 370 MHz (‘High Freq.’), with 10 dB power attenuation applied in the Low Freq. measurements. Some preliminary experiments were performed in the 0 - 400 MHz range (with 10 dB attenuation). Orientation selection of some degree, as in point 3 above, is inescapable even in simpler non ordered systems nominally resembling the OEC, such as mixed valence Mn\textsuperscript{III} Mn\textsuperscript{IV} dimers. However in such cases, it is sometimes possible to resolve largely non-overlapping transitions in the echo-detected envelope, corresponding to particular hyperfine values. ENDOR collected at these points is generally straightforward to interpret and simulate. Such points can be at the extreme edges of the absorption spectrum, where for instance, the $A_\perp$ components of Mn\textsuperscript{III} appear (largely in isolation). These possibilities are generally unavailable or impractical here. The ML signal shows nothing like the resolution of transitions with individual nuclear spin states across the envelope, as seen in model dimers, and is very indistinct and of low intensity near the edges. In preliminary experiments ENDOR spectra were obtained around regions labeled a, b and c in Figure 6.3. Typical data, for the Low freq. region are shown in Figure 6.6.
Figure 6.6 $^{55}$Mn Davies ENDOR spectra for the 0-140 MHz frequency range, acquired (2.5 K) at nominal points a, b, c on the ML envelope (see Figure 6.3, normalized to equivalent numbers of scans). The spectrum for point b is a combination of several data sets (same or different samples, see discussion in text, Fig 6.7 and and Fig 6.8 “ENDOR sampling point sensitivity”), taken on different experimental runs but at nominally the same g value (2.05 ± 0.01). This corresponds to an effective field range of < 3 mT. All data taken with 10 dB RF attenuation. Red spectrum is from point b for well annealed S1. Single starred peaks are from $m_I = \pm \frac{1}{2}$ related Mn II transitions and the doubled starred peak is a 1st harmonic peak from the $m_I = \frac{3}{2}$ related transition at ~ 340 MHz (see text and Fig 6.7). Transitions in the ~ 10 - 20 MHz region are expected to arise from protons coupled to the ML center (see text).
Figure 6.7 High frequency region (140 - 370 MHz) $^{55}$Mn ENDOR spectra at 2.5K, for representative samples, taken at point b on the ML envelope (0 dB RF attenuation). Red spectrum is from fully annealed $S_1$. Variation in the individual positions, intensities, of the strong transitions in the ~ 200-230 MHz between samples is evident (see text and fig 6.8 "ENDOR sampling point sensitivity") Starred peaks are from Mn$^{II}$, as in Figure 6.6.
- **ENDOR sampling point sensitivity**

![ENDOR spectra graph](image)

*Figure 6.8 shows two low frequency (~10 dB RF Attenuation, ~1000 scans each) ENDOR spectra for the same PS II sample, taken on two separate accumulations runs, at the same nominal field / MW frequency conditions (Field = 335.0 mT, Freq. = 9.656 GHz), but with sample adjustment and bridge retuning between each. The prominent peaks in the ~200-225 MHz region tend to occur in pairs, (203 and 219 MHz) or (212 and 223 MHz), with somewhat variable relative intensities. Interestingly, peaks at ~140-160 MHz shift with them, consistent with all these positions corresponding closely to quadrupole transitions predicted for the A₂ manifold of Mn 1 (see Figs 6.6 and 6.7, main text).*

The ENDOR spectra in Fig 6.6 and 6.7 are far sharper, with more resolved detail than any previously reported for the OEC S₂ ML signal. Resonances are seen across the full frequency range sampled, including features derived from the 'uncoupled' MnⅠ. These occur as very sharp features at well known (but slightly variable) positions around 101-105 MHz and 153-157 MHz (mₛ = ±½ derived) and at ~340 MHz (mₛ = 3/2 derived, e.g. see [55a, 56]). In addition, weaker transitions are sometimes seen (depending on RF power etc.) at precisely half the above frequencies, due to first harmonics generated by the RF frequency doubler (see
Methods). The relative intensities of features, notably the low (< 100 MHz) frequency resonances and the components around 200 MHz varies significantly with main field position, but all features seem to be represented with reasonable relative amplitude in spectra taken at the envelope peak (point b region, shows combination of several representative data sets). Most measurements were then made in this region. The results in total contrast sharply with all previously reported data on $^{55}$Mn ENDOR from the ML center (see Figure 6.9 and 6.10 below), which show, as noted earlier, a broad, partially resolved envelope between ~ 85-170 MHz (at X or Q band), with some structure but little if any systematic dependence of the ENDOR pattern on envelope sampling position, even with larger variation of the acquisition points about the central peak position than in Figure 6.3. As acquired our data typically exhibit some baseline slope or weak, very broad features. These may in part be real signal effects (see the data analysis-simulation section below), but at present we are not confident of being able to reliably isolate these. Therefore the data have been digitally leveled, where necessary (generally with linear baseline corrections).

The data in Fig 6.6 show that several weak, likely anisotropic Mn hyperfine couplings are present (~ 40 - 120 MHz) and so, as a consequence of the total ML width, large couplings must also be present. Fig 6.7 (High Freq. region) shows this to be the case, with resonances seen between 200 - 340 MHz. The spectrum from well annealed $S_1$ PS II complexes shows that most of these features must arise from the $S_2$ ML state, with only the expected Mn$^{II}$ features being present in the $S_1$ state samples. The sharp multiple peaks around 200 MHz and near 300 MHz and above are superficially characteristic of quadrupole transitions, presumably from the lower and upper edges respectively of a single large, highly anisotropic Mn hyperfine tensor, whose central component is responsible for the weak transitions.
around 250 MHz. Indeed the relative intensities within the closely spaced group of transitions near 200 MHz exhibit a remarkable sensitivity to field acquisition value, as the two spectra in Figure 6.7 were taken (on separate samples) at nominally equivalent field points in the b region (cf also the behaviour of these peaks in Figure 6.6). This behaviour of the intense ~200 MHz peaks was also seen in Low Freq. measurements in the b region, so they must arise from closely (< 1 mT) field spaced hyperfine positions in the ML envelope, which our sample/measurement conditions do not significantly broaden or smear.

Fig 6.9 55Mn Davies ENDOR spectra for the 0 -140 MHz frequency range, acquired at 4.2 K at nominal point b on the ML envelope (see Fig 6.3). All data taken with 10 dB RF attenuation. Red spectrum is from point b on well annealed S1 sample. Mn2 hyperfine range indicated, blue starred peaks are from MnII as in Figure 6.6 and 6.7. Shown also is the reproduced X Band 55Mn ENDOR spectrum from Messinger, Lubitz et al [59], for the ML spectrum from PS II membranes (+ MeOH) at 4.2 K (membrane narrow and broad).
Although several $S_2$ related ENDOR peaks are seen in the 90-170 MHz region, where all previous studies have located (broader) resonances, these clearly do not arise from near isotropic species. Nor do they totally dominate the ENDOR spectra, as in the previous studies.

To address further the possibility that this might be nuclear relaxation related, a more limited set of experiments were conducted on the PS II core material at higher temperature (4.2 K), equivalent to that used in all previously reported PS II $^{55}$Mn ML ENDOR experiments except those of Krewald et al. The results are shown in Figure 6.9 and 6.10. Now the resonances in the 90-170 MHz region become relatively more pronounced, while those around 200-220
MHz are diminished and essentially nothing beyond the Mn\textsuperscript{II} feature is seen at higher frequencies. For comparison, Fig 6.9 reproduces the S\textsubscript{2} X band $\text{^{55}Mn}$ ENDOR spectrum from Lubitz et al. [59], which is very similar to that reported earlier by Britt et al, and most recently by Krewald et al [55b]. The former is for PS II membranes in the presence of MeOH and differ slightly from the present PS II complexes (the membrane samples are probably narrow form like, see below), while the Krewald et al. data are for cyanobacterial PS II complexes. However it is clear that although the present spectra continue to be significantly more resolved than those from any previous study, the results start, generally, to ‘resemble’ those of earlier workers, both in the emergent shape of the spectra in the mid frequency range and suppression of features beyond ~ 200 MHz in the high frequency range. From Figure 6.3, the electron T\textsubscript{1} relaxation rates in the core samples, even at 4.2 K, are still at least an order of magnitude less than those likely to have existed in all previous ENDOR studies on the ML signal. It would indeed be necessary to use impractically high temperatures (> 10 K) to match the earlier conditions in this regard. At 2.5K the plant cores exhibit a T\textsubscript{1} relaxation for most of the sample that is still ~10 fold less than for the measurements of Krewald et al. at the same temperature.
6.2 DATA ANALYSIS-SIMULATION

6.2.1. Introduction

The complexity of the Davies ENDOR data from the ML signal revealed here, together with the resolved detail within the corresponding X Band CW spectra, present both opportunity and challenge for interpretation and simulation. Spin Hamiltonian parameter sets as simple as those that have been previously employed, with all tensors (hyperfine and g tensors) aligned and all hyperfine tensors exhibiting modest, at most, anisotropy, are inappropriate. Indeed in the high oxidation state paradigm almost universally assumed by earlier workers, there is little distinction between the anisotropy assigned to the single Mn\textsuperscript{III} in the cluster and some (at least) of the Mn\textsuperscript{IV} centers [60]. However, as the present results clearly indicate a ‘dimer like’ magnetic arrangement within the S\textsubscript{2} OEC cluster, the simulation approach has been based around one large highly anisotropic coupling, one medium (of order isolated ion values) coupling and two smaller sets of couplings, corresponding to the ENDOR resonances below 100 Mhz (and the resolved ‘super-hyperfine’ structure in the CW spectra). All tensors are formally permitted to be anisotropic, with no assumption that axes are aligned. The molecular axis is taken to be that of the largest hyperfine coupling and all other tensor orientations are referenced to that.

The spin Hamiltonian used for the ML cluster analysis formally includes all relevant nuclear terms, but assumes a strong coupling exchange limit between the Mn ions, with net electron spin, S, of 1/2. Consequently no exchange or fine structure terms are included for the electrons, only a net g tensor. As is well known [61], under these circumstances, each effective hyperfine tensor, $A_i$, of the individual (labeled i) Mn ions, is given by $A_i = \rho_i A_{\text{ion}, i}$,
where $\rho_i$ is the spin projection coefficient of the ion within the net $S = 1/2$ manifold. The $A_{\text{ion},i}$ is an ‘isolated ion’ value, somewhat hypothetical, which would be seen if the ion, in an ‘equivalent’ chemical environment (ligands etc.), were not magnetically exchange coupled to other ions. This is the quantity, which is obtained from a single determinant quantum chemical calculation on the cluster (e.g. in ADF, ORCA etc.). For the coupled system, the net $g$ tensor is similarly given by;

$$g = \sum_i \rho_i g_i,$$

where the $g_i$ values are those of the individual ions.

So the spin Hamiltonian becomes;

$$H = S \cdot g \cdot H + \sum_i g_n I_i \cdot H + \sum_i I_i \cdot A_i \cdot S + \sum_i I_i \cdot Q_i \cdot I_i$$

where $I_i$ is the nuclear spin, $Q_i$ the nuclear quadrupole tensor, $g_n$ the nuclear g value for $^{55}\text{Mn}$ nucleus $i$, ($i = 1...4$) and $H$ the external field.

CW simulations of the ML signal were performed in EASYSPIN, using the 2nd order perturbation theory option for all four Mn, or full matrix diagonalisation with three Mn. This means that, for consistency, quadrupole interactions were not included in these calculations, which has generally been the case in previous studies by others (e.g. [55a]), when dealing with systems of more than two coupled Mn centers. Full Hamiltonian diagonalisation becomes very unwieldy with so many Mn included. Preliminary calculations, on three Mn center systems, showed that inclusion of quadrupole terms of the magnitudes involved here (see below) had little overall influence on the simulated CW spectra at X band. However they possibly have some effect, as discussed below and detailed studies, using the SOPHIE [62] program suite are currently underway. These will be reported separately.
DFT calculations were first performed on isolated model OEC structures previously identified by Pace et al. as representing configurations consistent with available XRD data [63]. These were mostly to provide initial estimates of the relative orientations of hyperfine tensors for the four Mn within the Mn\textsuperscript{III, IV, III, III} configuration of the low oxidation state paradigm assumed here. The tensor principal components were fitted, largely by manual adjustment, to both the CW and ENDOR spectra, iteratively, as well as final adjustment of tensor relative orientations. Because this work concerns understanding features on the ~ 1 mT scale, consistent with the high resolution data, the line-width parameter used (1.5 mT) was significantly less than that which appears to have been employed by others (estimated as generally > 3 mT), using their reported hyperfine parameters).
Figure 6.11 Simulation of the ‘broad form’ (PS II Cores) ML spectrum (X band) from Fig. 6.1, using the parameter values in Table 6A. The discontinuous region near \( g = 2 \) in the experimental data corresponds to a TyrD subtraction artefact and is not part of the ML spectrum. Frequency, Table 6A.

Figure 6.11 shows a simulation of the ‘broad form’ (PS II Cores) ML spectrum (X band) from Figure 6.1, using the parameter values in Table 6A. Although not a perfect cosmetic fit to the experimental spectrum, the simulation reproduces, at least semi-quantitatively most of the characteristic detail of the broad form spectra. The deviation just to the low field side of \( g = 2 \) may reflect imperfect subtraction of the strong background features (TyrD etc.) in the \( S_2 - S_1 \) difference. The simulated spectrum shows more fine detail near the edges than seen...
experimentally and this is considered further below. The parameters for Mn 1 and 2 in Table 6A are regarded to be robust, from the fitting and those for Mn 3 to be reasonable, although there has been no attempt to fit a quadrupole term for this ion, which could be relatively significant since the effective hyperfine tensor is not large. The parameters for Mn 4 are merely indicative and indeed simulations using Mn 1,2,3 only (plus quadrupole for Mn 1) give results hardly different (not shown but see Fig 6.12 below). Thus Mn 4 is virtually ‘switched off’ while both Mn 1 and 3 are highly anisotropic and almost totally rhombic. Even Mn 2, which in the low oxidation scheme would be in the IV state, has significant (for MnIV) rhombic anisotropy. It is not yet clear that the fitting is truly ‘optimal’, there are a substantial number of free parameters, but many of these (hyperfine tensor principal magnitudes) are significantly constrained by ENDOR (see below).

Table 6A: Simulation Parameters for X-Band, CW, Multiline, ‘broad-form’

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<td>1.964</td>
<td>1.987</td>
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<tr>
<td>A_{Mn1}(MHz)</td>
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<td>-337</td>
<td>-514</td>
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<tr>
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</tr>
<tr>
<td>A_{Mn4}</td>
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<td>-1</td>
<td>-7</td>
<td>&lt;</td>
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<tr>
<td>Euler angles (Rad.)</td>
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<td>b</td>
<td>g</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>2nd Order Perturbation Theory</td>
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Figure 6.12 Simulation of the ‘narrow form’ (PS II membranes) ML spectrum (X band) from Figure 6.1, using the parameter values in Table 6B. The region near \( g = 2 \) in the experimental data corresponding to the Tyr\( \beta \) background signal subtraction has been omitted. Frequency, Table 6B.

Figure 6.12 shows the corresponding simulation of the ‘narrow form’ (PS II membranes + glycerol) ML spectrum (X band) from Fig. 6.1, using the parameter values in Table 6B. The fit is quite reasonable and the contribution of Mn 3 is now \( \sim 60\% \) of that for the broad form, while retaining very similar hyperfine parameters to the latter for Mn 1 and 2. This is precisely the qualitative conclusion derived above in the discussion of the data in Fig 6.1. Mn 4 is essentially absent and the simulation in fact does not include it at all. The principal changes between the narrow and broad forms are centred around Mn 2 and 3, with mainly a tensor
rotation for Mn 2 (more closely aligned in the narrow form with Mn 1) and Mn 3 being closer to ‘switch off’ in the narrow form. A hyperfine contribution from Mn 4 is not detectable in CW modeling for the narrow form. The g tensors are indistinguishable between the broad and narrow forms at X band, but certainly different from the values found by Cox et al. [64] for thermophilic PS II, principally in the $g_x$ and $g_y$ values, which are smaller (by $\sim 0.010 - 0.016$) than found here for spinach cores (even at X band these differences result in spectral shifts of $\sim 2$ mT which are readily distinguishable from our data (e.g. see Figure 6.2).

Table 6B: Simulation Parameters for X-Band, CW, Multiline, - ‘narrow-form’

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<td>$A_{Mn2}$</td>
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6.2.3. X-Band Davies ENDOR simulation

- “Low field”

Figure 6.13 Simulation (solid red) of the low frequency region PS II core ENDOR spectrum from Figure 6.6 (point b), using the parameter values from Table 6C (ENDOR). Calculation includes only contributions from Mn 2, 3 and 4 and describes the resonances below ~170 MHz. These extend down into the 100-150 MHz region. Expected proton region indicated.

Figure 6.13 shows a simulation of the PS II Core ENDOR spectrum from Figure 6.6 (point b), using the parameter values from Table 6C (ENDOR). This is calculated for a full powder pattern using only the parameters for Mn 2, 3 and 4 and describes resonances below ~170 MHz. In addition, an approximate simulation (see below) of Mn1 contributions in this region is also included (dashed). Relative scaling between these two is necessarily arbitrary and that shown is deemed reasonable from the experimental spectra. Together they are clearly quantitatively (within ~5 MHz) consistent with the data in terms of frequency positions, but
intensities are qualitative only. The EASYSPIN ENDOR simulation procedure includes quadrupole interaction, but calculates a simple powder pattern average for the nuclear transitions, which are assumed to be uniformly sampled orientationally at the measurement point. That is almost certainly not the case for the highly resolved and anisotropic nature of the electron spin packets sampled in the ML measurements, (particularly for Mn 1 contributions), although the approximation becomes better for the weaker couplings (Mn 3,4) which are expected to contribute a range of orientations at any given sampling point near the ML center. The strong transitions above 200 MHz clearly arise from Mn 1, but interestingly this ion also contributes variably (along the A$_z$ manifold, see below) in the ~ 100-150 MHz region.
“High field”

Figure 6.14 Simulation (red) of the high frequency region PS II Core ENDOR spectrum from Figure 6.7 (point b), using the parameter values from Table 6D (ENDOR). Calculation includes only contributions from Mn 1 and describes the resonances in the region ~ 170 - 360 MHz.

The assumption of powder pattern averaging is very poor for the high frequency ENDOR resonances arising from Mn 1, whose coupling and anisotropy is the largest yet seen for a Mn\(^{\text{III}}\) center, to the author’s knowledge (see next sub-section “ML Envelope shapes for Mn1 Principal Axis Directions”). The simulation problem becomes particularly challenging under these conditions and no attempt at a formally exact treatment is given here. However, an approximate analysis which takes some account of the particular circumstances applying for Mn 1 is readily possible. This is also consistent with the baseline levelling of the data used...
here (see above for baseline levelling). Firstly, because the A tensor for Mn 1 is almost purely rhombic (i.e. $A_{\text{iso}} \sim A_Y$), each principal axis value is almost equally likely to be viewed along a given orientation within the powder pattern (e.g. not the case for near axial symmetry, as typically found for Mn$^{\text{III}}$). It is assumed then that the relatively sharp turning points associated with these directions are the prominent features in the experimental ENDOR data. Further, the sampling position near the ML centre means that, given the magnitudes of the other Mn couplings (particularly Mn 2, $A_{\text{iso}} \sim 230$ MHz), only ENDOR transitions from $|m_i| = \frac{1}{2}$ on the $A_X$ manifold are likely to be seen (see sub-section “ML Envelope shapes for Mn1 Principal Axis Directions”). Transitions from all (or almost all) $m_i$ values on the $A_Z$ manifold should be seen, while for $A_Y$ the situation is intermediate. However for simplicity all these transitions are included. Further the local density of transitions (i.e. likelihood of detection) at a sampling point near the pattern centre should be, roughly, inversely proportional to the principal axis $A_i$ value ($i = x, y, z$) for Mn 1 (sub-section “ML Envelope shapes for Mn1 Principal Axis Directions”). Figure 6.14 then shows such a simulation of the PS II Core ENDOR spectrum from Figure 6.7 (point b), using ENDOR parameter values from Table 6D. This is calculated using the Mn 1 parameters only. Again, there is a reasonably good agreement with the data, in regard to frequency positions for most features, but also, again, some intensities are not well reproduced, with a few transitions (notably from the $y$ direction, sub-section “Origins of simulated ENDOR transitions”) exaggerated. However the intense peaks in the 200-225 MHz region are shown clearly to arise from the quadrupole manifold of $A_Z$ (see also Figure 6.13) which may be variably selected with precise observation point, while those above 300 MHz come mainly, as expected from the $A_X$ manifold. Slightly different values of the Mn1 hyperfine terms are estimated from the CW and ENDOR fits (Table 6A, B, C, D) and we
suspect that there is some spread of this (and presumably the quadrupole value) within our samples. Simulations show that this would lead to ‘blurring’ of the fine detail seen in the simulations but not experimental data for the broad form CW pattern near the spectral edges (cf Figure 6.11). Further, the assumption is made in the Figure 6.14 simulation that the quadrupole and A tensors are aligned for Mn 1, which for a centre of its unusual nature is unlikely to be strictly true and this may account for some of the (slight) deviations of the predicted peak positions from the data. Nonetheless, all of the above re-enforces the conclusion that the intrinsic line broadening in the samples at 2.5 K is small, as the 25 ns microwave pulse inverts electron spin packets that are only ~ 0.2-0.3 mT wide on the field axis. As discussed above, the very broad intensity variation predicted by the powder pattern simulation could not be reliably detected in our measurements and indeed the intensity in the experimental ENDOR spectrum is distributed in a manner suggesting pronounced (and essentially uncontrolled) orientation selection (sub-section “Baseline Uncorrected ENDOR data and EASYSPIN Powder Simulation”).

The degree of anisotropy exhibited by the Mn hyperfine parameters in Table 6A, B, C, D are significantly greater (both for the inferred Mn\textsuperscript{III} and Mn\textsuperscript{IV} centers) than that seen in dinuclear Mn\textsuperscript{III,IV} model compounds, including catalase. Lubitz, Bittl and colleagues have examined a number of such cases [33b,c]. In these the Mn\textsuperscript{III} center is expected to have a (strong coupling limit) projection co-efficient of $\rho_{\text{III}} = 2.0$ and the Mn\textsuperscript{IV} center a value of $\rho_{\text{IV}} = -1.0$. While showing significant rhombic character in some cases, the Mn\textsuperscript{III} centers are generally not too far from axial symmetry while the Mn\textsuperscript{IV} centers are typically not far from isotropic. However, the range of $A_{\text{iso}}$ values seen, vis ~ 400-450 MHz for Mn\textsuperscript{III} and ~ 200-240 MHz for Mn\textsuperscript{IV}, are not inconsistent with the values in Table 6A, B, C, D. On the basis of these,
estimates of the spin projection factors are indicated in Table 6A, B, C, D. Details of the spin-projection coefficient are provided below.
Figure 6.15 shows the simulated ML absorption envelope shapes for x, y, z orientations (relative to the external field) of the three principal direction hyperfine tensor on Mn 1 (i.e. molecular axis). Shown also are stick patterns showing the corresponding Mn 1 hyperfine transition field positions alone (no quadrupole). Since the maximum width of the Mn 2, 3, 4 splitting pattern is only ~ 50 mT, only transitions for Mn 1 involving $m_I = \pm \frac{1}{2}$ are likely to be sampled along the x direction, whereas all (or nearly all) such transitions will be sampled for the z direction. The y direction contributions will be intermediate, but less readily defined in total.
"Origins of simulated ENDOR transitions"

Figure 6.16 shows the principal axis direction origins (x, y, z) of the transitions used to construct the simulated data in Figure 6.14.
Figure 6.17 shows an example of baseline uncorrected ENDOR amplitude data in the high frequency region, together with a full powder pattern simulation of this region using Mn1 parameters from Table 6D. It seems likely that the raw data actually contain some elements of the powder pattern shape, although these are substantially distorted by orientation selection.
### Table 6C: Simulation Parameters for X-Band, Multiline, ENDOR, ‘low-frequency’

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<th>Z</th>
<th>$A_{\text{iso}}$</th>
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<td>2.008</td>
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<td>$A_{Mn2}$</td>
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<td>$A_{Mn3}$</td>
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Linewidth (mT) | 2.0
Exp. Field (mT) | 338.5
Method | Complete Matrix Diagonalisation

### Table 6D: Simulation Parameters for X-Band, Multiline, ENDOR, ‘high-frequency’

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Linewidth (mT) | 3.0
Exp. Field (mT) | 339.5
Method | Complete Matrix Diagonalisation
The results obtained here for the spin Hamiltonian parameters of the $S_2$ ML state have significant implications for the nature and magnetic organization of the Mn ions within OEC cluster. Firstly, the observation of large (in fractional terms) anisotropy of two, probably three Mn centres, with modest anisotropy in the remaining centre (near rhombic in all cases), strongly suggests that three Mn$^{III}$ ions and one Mn$^{IV}$ ion are present, i.e. the low oxidation state paradigm prevails. Identification of only two Mn$^{III}$ species is of course sufficient to ensure this. Although the validity of the low oxidation paradigm is not currently a majority view (e.g. see [8b]), it has been shown, using Time Dependent DFT, that when the metal ligand environment is accounted for, the available Mn XANES data are most consistent with this picture [65]. A recent re-examination of other spectroscopic data also supports this conclusion [20], as does a recent experimental study by Dismukes et al. [66], that counts the electrons removed from four Mn$^{II}$ ions in photo-assembly of the functional enzyme (from the apo-protein). Finally, a very recent large scale computational study of the OEC region [14] was able to rationalize detail of both the 2.9 Å and 1.9 Å XRD structures [7e) f]), within the low oxidation state paradigm, without the need to invoke radiation damage or photo-reduction.

A second notable and somewhat surprising outcome is that the magnetic coupling in $S_2$ is actually ‘dimer like’, i.e. two Mn ions are almost magnetically ‘switched off’. This was first proposed empirically by Åhrling et al. [4] and the possible basis for it has been discussed more recently from a computational chemistry viewpoint [19]. The situation is illustrated in Fig 6.18A, using the conventional Mn numbering scheme of Pace, Stranger et al. [67] in which Mn1 is ligated by His 332 and Mn 4 (‘dangler’) by Asp 170. In the low paradigm $S_2$, the
oxidation states for Mn 1..4 are respectively; III, IV, III, III. The net coupling within the Mn 1-2 and Mn 3-4 pairs is substantially anti-ferromagnetic (J ~ -30 to -50 cm⁻¹) from preliminary calculations [19], although these are now being pursued at a more extensive level and will be reported separately. The net coupling between the dimers, mediated principally by the Mn 1-3 and Mn 2-3 interactions, is variable within structures examined by Pace, Stranger et al. and might be close to zero in special circumstances, corresponding to the precise relative orientation of the dimers around the Mn 2-3 ‘hinge’. There is indeed a clear ‘hint’ of this in the simulation results for the broad and narrow ML forms above, with the major differences occurring on Mn 3 (Table 6A and 6B). Then the Mn 1-2 pair closely resembles a classic III-IV dimer (spin projections coefficients, \( \rho_1, \rho_2 \sim 2 \) and -1 respectively), while the Mn 3-4 pair is largely magnetically silent (both projections small or \( \sim 0 \)). This appears to be the case and fully rationalizes a series of elegant earlier experiments, mostly by Britt, Debus and co-workers, which may be interpreted to have probed the spin projections on three of the Mn centers (Mn 1,2,4) by ligand ENDOR/ESEEM, now that the crystal structures are known. This has been suggested previously [24d] but is summarised for convenience again here. Britt et al have determined [34a,b] that the single strong ^14N coupling seen in the S2 ML signal (from the ligating N of His 332), has \( |A_{iso}| \) values of 7.3 (spinach) and 6.95 MHz (syneocystis PS II.). A more recent determination (on thermophillic PS II) with W band EPR gives this as 5.9 Mhz [64]. For all computational structures of the hydrated S2 state that we have examined (and generally, even by others within the high paradigm [19]), the imidazole nitrogen ligand to Mn 1 interacts with the singly occupied metal (oxidation state III) \( t_{2g} \) based orbitals through \( \pi \) interaction only. ENDOR and ESEEM studies on model Mn complexes containing aromatic nitrogen ligands, including Mn catalase ([68] and references therein), have shown that the
hyperfine coupling of the ligating N is mostly isotropic. Further, for such π interaction with 
MnІІІ or MnІV, the single ion 14N |A_{iso}| value is consistently ~ 2-4 MHz. For σ ligation (to MnІІІ, 
through singly occupied e₈ orbitals, usually axial), the |A_{iso}| value is substantially larger (> 9 
MHz). Thus for Mn1, with π bonding to the His N and with 14N |A_{iso}| ~ 6-7 MHz, ρ must be ~ -2.

Debus, Britt et al. [68b, 35] have shown that for the cyanobacterial mutant in which D170 
(which ligates Mn4) is replaced by histidine, the mutant retains near normal photosynthetic 
function, with S₁, S₂ EPR signals indistinguishable from the native enzyme. Further no 
additional nitrogen coupling to the ML signal is detectable by ESEEM in S₂. However during 
the initial stages of photo-assembly, when the Mn ligated by D170 binds as a single MnІІ, 14N 
coupling to that Mn is readily detectable in the D170H mutant. While other interpretations 
cannot be totally excluded, the simplest is that a histidine at the 170 position on the D1 
peptide does bind Mn 4 in the assembled, functional system, but that ρ ~ 0 for the ion in that 
case.

This group [70] has also recently examined the interaction of the 13C labeled α carboxylate 
carbon of the D1 C-terminus (A344) with the S₂ ML state. The carboxylate is a bidentate 
briding ligand between Mn 2 and Ca in the 1.9 Å and 2.9 Å structures. Britt et al. found that 
the most consistent interpretation of the observed 13C dipolar plus contact hyperfine 
interaction with the S₂ Mn cluster was obtained assuming the carboxylate O ligated a Mn 
with  ρ ~ -1, and was adjacent to a single Mn with +1 < ρ < +2 . This yields a completely 
consistent picture of the coupling in the cluster if the latter Mn center is taken as Mn 1. Since 
the four Mn projection coefficients must sum to unity and ρ₄ is small, ρ₃ must also be small.
These conclusions regarding the OEC Mn spin projections also have important implications for the interpretation of other magnetic properties of the $S_2$ state. In particular, hyperfine interactions with ligands, including exchangeable waters (substrate and otherwise bound). Two of the Mn centers (Mn 3,4), which from crystal structures [7e] and modeling have water molecules bound or nearby (in lower $S$ states at least), are virtually ‘switched off’ magnetically. While counterintuitive (see below), this allows a convenient simplification in interpreting the OEC site magnetic parameters, which is given here. Detailed computational investigations are in progress and will be reported separately.

![Figure 6.18](image.png)

*Figure 6.18: A Magnetic coupling scheme for the $S_2$ OEC, based on DFT calculations of model OEC clusters [9], which would be consistent with the EPR studies on the ML system reported here. Mn numbered by the convention in [67]. Small values or near net cancellation of the $J_{23}$ and $J_{13}$ terms would need to occur. Then the system approximates two isolated dimers, one with net spin $\frac{1}{2}$ and the other with net spin $\sim 0$. B: Structural model of the OEC ($S_2$ state), based on the 1.9 Å XRD structure of Kamiya, Shen et al. [7e] and recent computational modeling of this structure by Gatt et al. [14]. The O(5) species has been identified as a water molecule [14], as is the W1 species, but W2 is likely an hydroxide in $S_2$ [52]. The molecular axis system, consistent with our data and that of Lubitz, Cox et al [64] is shown. This has a $z$ axis essentially along the Mn 1 – O(5) direction. The $y$ axis points along the Mn 1 – N(His 332) direction. See text.*
Cox, Lubitz and colleagues have recently presented an extensive study of the S$_2$ OEC at W band frequencies [64], including remarkable $^{17}$O labeled water exchange experiments using thermophyllic cyanobacterial PS II. As noted earlier, their resolved g tensor values are similar to those found here for spinach, but distinguishable. They find that the unique axis of the single $^{14}$N tensor seen on the W band ML signal, which points essentially along the Mn - N(His) bond [68b], is approximately parallel to the g tensor axis of largest value. That is the y molecular axis in the present scheme (Table 6A, B, C, D), which then means that this axis is oriented along the Mn 1 – His 332 bond (see Figure 6.18). Further, since Mn 1 (with $\rho \sim 2$) is the only Mn$^{III}$ ion of magnetic significance in the coupled cluster and Mn 2 is in the IV state (with $\rho \sim -1$), Mn 1 is expected to dominate the g anisotropy of the whole cluster. Thus the lowest g value direction (z) should correspond with the Jahn –Teller axis direction on Mn 1 and then the molecular x axis is also determined (see Figure 6.18). In rapid mixing experiments with $^{17}$O labeled water, Cox et al. found three classes (strong, intermediate, weak) of $^{17}$O interaction with the ML center in S$_2$. The largest (in magnitude, the tensor components are actually expected to be negative) $^{17}$O interaction seen was along the (smallest g) z direction. The interaction of a water molecule along the Jahn Teller axis of Mn 1 (i.e. along a Mn singly occupied t$_{2g}$ orbital direction), should be quantitatively similar to that of any of the equivalent Mn-H$_2$O interactions in hydrated Mn$^{II}$. For these the hyperfine interaction has been determined in detail [37]. Although the rhombicity in these cases is less than for the above strongly coupled OEC $^{17}$O, the unique direction, with largest magnitude coupling, points along the Mn - O bond (as expected from symmetry). The $|A_{iso}|$ value for the $^{17}$O in the hydrated Mn II cases is 7.4-7.5 MHz, while Cox et al. found a value of 9.7 MHz for the strongly coupled $^{17}$O. Our simple arguments would predict a value $\sim$ twice 7.4 for the
OEC, but it is likely that the Mn 1 - O bond length in the OEC is slightly longer than in hexaquo Mn\textsuperscript{II} say. (~ 2.3, vs 2.1 Å, see below). So semi-quantitatively (and totally qualitatively), the recent data from the Lubitz laboratory on the molecular g tensor and \textsuperscript{14}N, \textsuperscript{17}O hyperfine interactions with the Mn cluster in S\textsubscript{2} are completely consistent with the results obtained here and the model in Figure 6.18. Further, all these data are totally consistent with the earlier ESEEM determination, by Evans, Åhrling et al., [57a] of a single exchangeable (likely substrate) water dominating the exchangeable proton (or deuteron) interactions with the S\textsubscript{2} (and S\textsubscript{0}) Mn cluster. This required an interaction of the water deuterons with (effectively) a single Mn ion in the cluster, possessing a \( \rho \) value of ~ 2. This then lead to an inferred Mn-O(water) bond length of ~ 2.3 Å, compared to a length of ~ 2.1 Å estimated from the dipolar part of the \textsuperscript{17}O interaction seen by Cox et al. (2.2 MHz). It seems reasonable that within experimental-interspecies variations/uncertainties etc., these results be regarded as equivalent.

An intriguing observation concerns the nature of the quadrupole interaction on Mn 1. Because the Mn 1 hyperfine tensor is almost certainly negative (as for the isolated ion), the sign of the quadrupole tensor is then determined by the best fit to the ENDOR data (high and low frequency). The quadrupole tensor is not too far from axial, but oriented with the negative axis along x (not z, Table 6D). This means that the quadrupole is dominated by paired, not unpared spin density (ie back bonding from ligands into nominally empty Mn 3d, 4p orbitals).
Cox et al. interpreted their data within a currently popular picture in which the oxy species occupying the O(5) equivalent position in the 1.9 Å crystal structure is presumed to be an oxo bridging two Mn, - even in S₁. [71]. This is largely a consequence of an assumed high oxidation state paradigm for the OEC Mn and the presumption that the XRD structures (nominally S₁) have been perturbed by photo-reduction [72]. However, it has recently shown [73] from large scale modeling of the OEC and surrounding protein components, that the O(5) species is actually a water molecule, located on the approximate intersection of Jahn-Teller axes on Mn1,3,4 (it is close to the water position in Fig 6.18). These Mn (in the low oxidation paradigm) retain the III state in S₂ and calculations (now ongoing) indicate that the water molecule retains its approximate position, although the local potential well is shallow. This is also supported by earlier studies on smaller model structures [67b, c]. In the rapid mixing experiments reported by Lubitz, Cox, et al., complete exchange of labeled water with the site (in dark S₁) occurred in less than 15 sec. Even allowing for the temperature of the process (20°C), the well documented water exchange data of Hillier, Wydrzynski and colleagues [10] make it very likely that, of the two exchangeable substrate waters (‘fast’ and ‘slow’), the only species likely to exchange completely on this time scale is the ‘fast’ water (particularly in S₁, where the ‘slow’ water exchanges two orders of magnitude slower than in other S states). This ‘fast’ rate is so rapid that it is virtually un-resolvable in S₁. And it is precisely in the O(5) like position that earlier calculations by Pace, Stranger et al. have located the fast water to be [67b, c]. Further, the closest experimental model we probably have for oxo bridge exchange, as invoked by Cox et al. above, is that of the bridges in super-oxidised catalase (containing a MnIII – (µO)₂ – MnIV center with similar ligation to the OEC). Here the
rate measured by Brudvig, Scholes et al. is \( \sim 1000 \) times slower \([73]\) than that for the presumed PS II oxo bridge.

So how did this circumstance come about? The major culprits, as far as can be judged here, are two;

1) The fact that under all published experimental conditions employed before the current work (sample preparation type, measurement temperature etc.), the three \( S_2 \) OEC Mn ions in the III oxidation state were virtually undetectable in \(^{55}\text{Mn ENDOR}, \) due to nuclear relaxation effects. Essentially the only ion seen was the single Mn\( ^{IV} \) (ie Mn 2). Although this ion is significantly anisotropic relative to typical model compound Mn\( ^{IV} \) species, even catalase (all near isotropic, see above), the ENDOR resonances due to it alone are all contained within a hyperfine range of \( \sim 180-280 \) MHz. Since absolute signal quantitation in ENDOR (ie the number of Mn contributing) is not generally possible, due to the highly indirect methods of detection employed (pulsed or CW), it was natural to infer that this observed ENDOR envelope represented all the Mn in the cluster. Then the fact that the resonances from these were all contained within a modest range, distributed around the known isolated ion \( A_{iso} \) value of \( \sim 200-240 \) MHz (Mn\( ^{III} \) or Mn\( ^{IV} \)), meant that all ions must have projection coefficients of order unity (magnitude), with no more than one with significant anisotropy \([60]\). This is necessary to be consistent with the CW ML pattern width \([9]\) and the anisotropic center was taken as the single Mn\( ^{III} \) in a presumed Mn\( ^{III} \), (Mn\( ^{IV} \))\(_3\) oxidation state assignment for \( S_2 \). Because the main peak spacing (\( \sim 9 \) mT) in the CW ML spectrum is close to a single Mn\( ^{IV} \) ion \( A_{iso} \) hyperfine value (ie \( \sim 220 \) MHz), such
schemes lead readily to reasonable simulations of the CW pattern if fine detail is ignored (typically the case).

2) The further fact that two of the Mn ions (both Mn\textsuperscript{III}) in the S\textsubscript{2} ML cluster are almost ‘switched off’, through exchange coupling, irrespective of any relaxation effects. This appears truly bizarre, as it seems to be devoid of obvious mechanistic purpose\textsuperscript{2} and is a ‘fluke’ of the precise geometry around the Mn2-O-Mn3 single oxo bridge [14, 19]. However it has important (and confusing) spectroscopic consequences. Thus the several protons on the two water/hydroxyl groups (W1, W2), known now from the 1.9 Å structure to be located on Mn 4, are only very weakly coupled to the ML center in ENDOR/ESEEM. Also of course the terminally bound oxy groups themselves, if exchanged with \textsuperscript{17}O, (reasonably rapid, probably minutes time scale [73]), would be largely ‘invisible’ on the ML by these techniques. In fact the only waters which interact significantly (through hyperfine etc.) with the ML center are those at or near the O(5) position (Figure 6.18). This is in part the reason why Cox et al. had to invoke a deprotonated species for this O(5) position, to keep the total number of proton interactions ‘reasonable’. It also explains the seemingly strange observation of ‘dry’ ML centers, seen by Evans et al. with D\textsubscript{2}O exchanged PS II (e.g. see discussion in [11]).

It has already been shown computationally that some readily energetically accessible configurations of the S\textsubscript{2} state Mn cluster exclude a water molecule from close approach to Mn 1 [67b,c], while of course having water/hydroxyl groups bound to

\textsuperscript{2} It certainly cannot be the case that Mn3 and Mn4 are ‘switched’ off in the g4 form of S\textsubscript{2}. This is a ground state with spin 5/2 or greater. However, the pioneering studies of Zimmerman and Rutherford, over 25 years ago, showed that this form of S\textsubscript{2} functionally cycled, in flash turnover, almost precisely as did the ML, spin ½ form [41]. See also discussion of g4 state in [10] and computational spin ladders for S\textsubscript{2} in [24].
Mn 4. The most recent work (still on S1 [14]) suggests that ‘intermediate’ states are also possible, with the O(5) water now ~ 3 Å from Mn 1. This would place it near the separation (inferred from a dipolar coupling of ~ 0.6 MHz [64]) of the ‘intermediate’ O species seen by Cox et al. It has already been stressed repeatedly, both on experimental [57b] and computational [67] grounds, that the functional ML center can be heterogeneous in spectroscopic properties. Thus the appearance of two ‘close’ O species in the studies of Cox et al is likely to be a further manifestation of this effect.

If either of the above had not obtained, it seems likely that the detailed nature/magnetic coupling etc, in the ML cluster would have been essentially ‘sorted’ years ago. This has been an example of Nature performing in what seems to be an almost devious manner. There were important clues early, with high frequency ENDOR resonances seen in the Kammel thesis from Berlin (Bittl, Lubitz) and the remarkable discovery by Britt, Debus et al. that the D170H mutant, while still fully functional (with a ML signal indistinguishable from wild type), gave no detectable additional 14N couplings in ESEEM [69]. Subsequent work excluded this being artefactual [68b]. But this was apparently insufficient, possibly because a number of the mutation studies (typically examined by FTIR difference spectroscopy), have proved difficult to rationalize within any paradigm (including ours) [74]. However this is clearly an important area to be addressed in the future and studies on the topic are currently underway.
CHAPTER VII CONCLUSION

Observations at 2.5 K of previously unobserved $^{55}$Mn ENDOR frequencies on the ML signal of PS II, corresponding to hyperfine couplings in the range $\sim 50$ to $\sim 680$ MHz, mean that the magnetic organization of the Mn ions in the $S_2$ state of the OEC is substantially different from previously thought. The new ENDOR data, together with the well established EPR shape of the ML signal, mean that two Mn contribute most of the ML pattern width and that the other two Mn ions in the cluster are effectively magnetically isolated and near ‘silent’. The only plausible arrangement consistent with this is that the dominantly visible Mn act, in first approximation, as a (relatively) strongly anti-ferro-magnetically coupled Mn$^{III,IV}$ pair, while the other pair, both in the same oxidation states (III or IV), are similarly coupled silent (spin zero). The net exchange interaction between the pairs is weak, but presumably variable, permitting one ion in the ‘silent’ pair to contribute variably (always $< 20\%$ of isolated ion value) within the total observed ML hyperfine spectrum. The contribution from the fourth Mn appears to be virtually zero (always $< 5\%$ of isolated ion value). These factors together are the probable explanation of a long known variability in the fine substructure associated with the ML signal, depending on details PS II species, biochemical preparation etc., in samples that are all enzymatically active in water oxidation.

Although little can be said of the precise hyperfine nature of the fourth Mn (apart from its general magnitude), the third, variably expressed Mn hyperfine interaction has high asymmetry, being almost purely rhombic. It is very similar in this respect to the dominant Mn of the strongly visible pair, which on general grounds has to be a Mn$^{III}$ species. So the data, at this level, favour the presence of two Mn$^{III}$ ions in the $S_2$ state, which means there would be three, i.e. the low oxidation state paradigm exists for the OEC. However it is known,
in high valent oxo bridged Mn dimer systems, that transfer of spin character through d orbital overlap (from Mn$^{\text{III}}$ to Mn$^{\text{IV}}$) can occur [43], making a Mn$^{\text{IV}}$ ion appear more magnetically anisotropic. This means that one could probably not exclude some such mechanism operating (say from Mn 1 to Mn 3) within a system as complex as the OEC- ie that both Mn in the near silent pair were Mn$^{\text{IV}}$, implying now the high oxidation state model for the cluster.
Reference


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