A PULSED EPR INVESTIGATION OF THE HYDROXYL

$^1$H NUCLEUS OF MYOGLOBIN HYDROXIDE

A Thesis in
Chemistry

by

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ABSTRACT

Pulsed EPR methods have been widely used to characterize the spin environments of bioinorganic cofactors. One of those cofactors is the heme prosthetic group that is found in many biologically important systems such as globins, cytochrome P450s, peroxidases etc. Due to its availability and stability myoglobin is an excellent model heme system. In the effort to characterize a heme spin system and compare the different magnetic resonance techniques, we have undertaken an investigation of myoglobin hydroxide to determine the hyperfine tensor of the hydroxyl proton and in turn estimate the Fe-O distance of the cofactor. In this study, the two- and three-pulse ESEEM, HYSCORE, Davies ENDOR and DFT calculations will be used to elucidate the $^1$H hyperfine tensor of interest and give insight in to the application of the point-dipole approximation used in magnetic resonance.
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<td>CW</td>
<td>Continuous Wave</td>
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<tr>
<td>ENDOR</td>
<td>Electron Nuclear Double Resonance</td>
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<tr>
<td>EPR</td>
<td>Electron Paramagnetic Resonance</td>
</tr>
<tr>
<td>ESE</td>
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<td>ESEEM</td>
<td>Electron Spin Echo Envelope Modulation</td>
</tr>
<tr>
<td>FID</td>
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Chapter 1: Introduction

1.1 Introduction and classical description of EPR

Electron paramagnetic resonance (EPR) is a spectroscopic technique that allows the detection of species containing unpaired electrons. The general principle of this technique is the resonant absorption of microwave irradiation by a paramagnetic substance.\(^1\) This absorption is detected when frequency of the irradiation matches the energy difference between degenerate electron spin levels of the system. The lift of degeneracy of these electron spin energy levels is in most cases due to the application of an external magnetic field, although in some cases (e.g. molecular magnets) the resonance, due to solely intermolecular interaction, could be detected even without this external perturbation. In classical terms, the electron of the system can be viewed as a fast spinning particle with an angular momentum \(\mathbf{S}\). A net dipole moment of such substances is associated with this angular momentum and can be expressed in the following way:\(^1\)

\[
\mu_e = \gamma S \quad (1.1.1)
\]

where \(\mu_e\) is the magnetic dipole moment, \(\gamma\) the gyromagnetic ratio and \(S\) the associated angular momentum. The gyromagnetic ratio for a free electron is given as:

\[
\gamma = -g_e \frac{e}{2m_e c} \quad (1.1.2)
\]

The constants \(e\), \(m_e\), and \(c\) are the charge and mass of an electron and the speed of light. The \(g\)-factor \(g_e\) is a dimensionless proportionally constant that correlates the angular momentum and the dipole moment of the paramagnetic particle. For a free electron in vacuum its value is \(g_e = 2.0023\), however, this value can vary significantly for more complex spin systems. The aforementioned net dipole moment readily interacts with the magnetic field just like two magnets next to each other. The magnets can be aligned in either parallel or antiparallel fashion, where the latter is lower in energy. In classical terms the Zeeman effect is the splitting of these two energy levels.

On the other hand, speaking in quantum mechanical terms, due to a presence of an unpaired electron with a spin \(\mathbf{S}\), the system’s energy ladder splits additionally into \(2S+1\) levels with the spin projection to the external magnetic field \(M_s\) varying from \(-S\) to \(S\) in integer steps. Application of a magnetic field removes the degeneracy of the spin levels in such a way that the stronger the field, the larger the energy gap between the different \(M_s\). A simple illustration of the Zeeman interaction is the behavior of a free electron with spin \(S = \frac{1}{2}\) system in a linearly increasing magnetic field \(B_0\). In Figure 1 are shown the two
quantum states $S = \frac{1}{2}$, $M_s = \pm \frac{1}{2}$, one representing spin up ($\alpha$), the other spin down ($\beta$). The resonant condition is met when the separation of the energy levels between $\alpha$ and $\beta$ matches the frequency of the microwaves (eq. 1.1.3).

$$\Delta E = h\nu = g_e\mu_B B_0 \quad (1.1.3)$$

where $\mu_b$ is the Bohr magneton, a unit of quantum mechanical angular momentum with the value of 9.2741 A m$^2$, and $B_0$ the strength of the laboratory magnetic field. Therefore, for a field of 3400G, the free electron resonant condition is met with photons of 9.5 GHz frequency. To achieve resonant absorption of the microwaves resulting in a transfer of spins of the system from one state to another it is necessary that the excited state is less populated than the ground state. Hence we arrive to one of the key equations in statistical mechanics called the Boltzmann thermal equilibrium expression that states that the population difference of two states is a function of temperature.

$$\frac{N_2}{N_1} = e^{\Delta E / kT} \quad (1.1.4)$$

$N_1$ and $N_2$ are the numbers of spins in the ground and excited states respectively, $\Delta E$ the energy difference of the two spin states, $k$ the Boltzmann’s constant and $T$ the temperature in Kelvin. At room temperature, most biological spin systems have population differences too small to be detected, in addition, liquid water readily absorbs large amounts of microwaves, which is one of the main reasons why most EPR experiments on bioinorganic systems are performed at cryogenic temperatures ($<77K$).

Another relevant feature of paramagnets in a magnetic field is their motion. It is a precession motion of the magnetic moment around $B_0$ called the Larmor precession (Figure 1.2) with its frequency similarly called the Larmor frequency $\omega_0$. Spins in different Zeeman energy level states precess in opposite directions. The ones in $M_s = +\frac{1}{2}$ state, by convention, rotate in a counter clockwise direction, while the spins in the $M_s = -\frac{1}{2}$ state rotate in the clockwise direction.
In the majority of cases, the resonance condition (eq. 1.1.3), in practice is not satisfied, reason being the interaction of the electron with its surroundings. To account for that, the constant $g_e$ in (1.1.3) is replaced with an orientation dependent value $g$, called the g-factor.

The interaction of the electron with its surroundings is quantum mechanically described by the so-called spin-orbit interaction. Therefore, the observed magnetic moment of most paramagnetic substances is not just due to the spin, but due to the coupling between spin and orbit magnetic moments. In Figure 1.3 is the vector representation of the spin and orbit coupling. Each Paramagnetic species, therefore, in an external filed possess a resultant magnetic moment $\mu_r$ (eq. 1.1.5) that arises from the coupling between spin (eq. 1.1.6) and orbit (eq. 1.1.7) magnetic moments:

$$
\mu_f = -g_f \mu_B J \\
\mu_S = -g_e \mu_B S \\
\mu_L = -g_L \mu_B L
$$

where $g_i = \frac{3j(j+1)+s(s+1)-l(l+1)}{2j(j+1)}$

In a magnetic field, the direction of the orbital magnetic moment is very dependent on the orbital symmetry of the spin environment. Therefore, very often, the magnitude of the magnetic moment is dependent on the orientation of the externally applied magnetic field. As a consequence, the g factor is generally expressed as a tensor that in a Cartesian space is represented with the following values:

$$
g = \begin{pmatrix} g_{xx} & g_{xy} & g_{xz} \\ g_{yx} & g_{yy} & g_{yz} \\ g_{zx} & g_{zy} & g_{zz} \end{pmatrix} \quad (1.1.8)
$$

where for example the $g_{xy}$ and $g_{xz}$ represent the y and z components that arise due to the x component of the magnetic field. This tensor is very often diagonalized yielding three principal g values that describe every EPR spectrum $g_{xx} g_{yy} g_{zz}$ or $g_x g_y$ and $g_z$. In biologically relevant samples, however, all molecular orientations are present and a powder spectrum is obtained. From (eq. 1.1.3) it is inferred that each field position of an EPR spectrum yields a unique g-value. This g-value, called the $g_{eff}$ in
a powder spectrum has contributions from all three principle g-values $g_x$, $g_y$, and $g_z$. As presented in Figure 1.4 the X, Y, and Z are the directions of the principle g-values and $\mu_{\text{eff}}$ is the effective spin magnetic moment. The $\theta$ and $\phi$ are the polar and azimuthal angles that define $\mu_{\text{eff}}$ in the principle g-value frame. Therefore, the observed, effective g-value is in terms of $\theta$ and $\phi$ is:

$$g_{\text{eff}} = \sqrt{g_x^2 \cos^2 \phi \sin^2 \theta + g_y^2 \sin^2 \phi \sin^2 \theta + g_z^2 \cos^2 \theta}$$

Although the most logical way to obtain a resonance spectrum is to sweep the microwave frequency and detect the absorption, this method is technically very challenging. The most common way to obtain an EPR spectrum, therefore, is to sweep the magnetic field while keeping the microwave frequency constant. Unpaired spins do not only interact with the magnetic field, but with their environment and amongst themselves. Therefore, the signal of a single resonant absorption line will have contributions from a range of applied magnetic fields. There are two line shape broadenings – homogenous and inhomogeneous broadening. Homogenous broadening in EPR spectra is always present. The absorption spectra have Lorentzian distribution character with the width determined by spin relaxation effects. Inhomogeneous broadening, as it can be inferred from the name, originates from the inhomogeneity of the system and is commonly described with Gaussian line shapes. The causes for such inhomogeneity could be due to special variation of Zeeman frequencies across the sample, anisotropy of interaction tensors in a power EPR sample, resulting in strain effects, specifically the g-strain.

1.2. EPR Spectroscopy – a Quantum Mechanical Phenomenon

Unlike in the macroscopic world where the energy of a system continuously fluctuates, quantum particles such as electrons have discrete states and correspondingly discrete energy levels. For any given energy value that the system can obtain, there is a corresponding quantum mechanical state. Each state is defined by a wave-function which is a function that contains information about the configuration of all the particles in a system of interest in this particular state. The square of the wave-function at a certain space point gives the probability of finding a quantum particle at that point. Obviously, an integral of the square of the wave-function $\psi$ is unity:

$$\int_{-\infty}^{\infty} \psi^* \psi \, dr = \langle \psi | \psi \rangle = 1$$

where $\langle \psi | \psi \rangle$ represents the Dirac notation. Quantum wavefunctions are separated into the spin and orbit part. The orbit part is commonly included in the spin orbit coupling phenomenon, while EPR spectroscopy is mostly concerned with the spin part of the wavefunction. Thus, for every experimentally measurable spin quantity there is a corresponding spin quantum mechanical operator. A specific
operator, operated on the wavefunction of interest will yield useful information that pertains to the system. However, in a single EPR sample, individual spins with slight differences in their environment are described with similarly different wavefunctions. Therefore, the physical quantities measured are average or expectation values represented in the following way:

\[
\langle P \rangle = \langle \psi_n | P | \psi_n \rangle = \int_{-\infty}^{\infty} \psi^* P \psi \, dr = \lim_{N \to \infty} \frac{1}{N} \sum_{i=1}^{N} p_i \tag{1.2.2}
\]

where \( P \) is the operator of interest, \( \psi_n \) the set of wavefunctions that describe the system, \( p_i \) the experimental value of \( P \) obtained from the \( i^{th} \) measurement and \( N \) is the number of measurements. Some properties of a spin system can be known and measured accurately like the square of the angular momentum and the energy. For example, the spin wavefunction is an eigenfunction of the energy operator; that is the operator applied to the function yields an eigenvalue, energy in our case, and returns the wavefunction. The energy operator is called the Hamiltonian and is represented by following expression:

\[
H \psi_k = E_k \psi_k \tag{1.2.3}
\]

where eigenvalue \( E_k \) is a unique energy that corresponds to the \( k^{th} \) state described by the eigenvector (wavefunction) \( \psi_k \). A convenient and succinct way to portray all the eigenstates of spin wavefunctions is via the matrix representation. If \( \psi_i \) and \( \psi_j \) are wavefunctions for discrete states where \( i \) and \( j = 1, 2, 3, \ldots \), and \( H \) is the Hamiltonian, every matrix element can be written as \( \langle \psi_i | H | \psi_j \rangle \). This way, the diagonal elements \( \langle \psi_i | H | \psi_i \rangle \) represent the average values of the energies of each eigenstate of the system, while the off-diagonal elements represent the mixing of pure states. The complete Hamiltonian of any physical system is very complex and contains many different terms that account for all possible interactions. In a general way, it can only be solved for a simple hydrogen atom using a state-of-the-art computing clusters available today.

For description of EPR experiments it turned out to be sufficient to use an effective Hamiltonian called spin Hamiltonian, in which only the nuclear and electron spin operators are written explicitly and all the other contributions are substituted by effective constants or completely omitted if the corresponding interactions is too large in comparison to the microwave frequency. Thus the energy of any paramagnetic species can be obtained via the following spin Hamiltonian:

\[
H = H_L + H_\text{Ze} + H_{\text{Hf}} + H_{\text{zn}} + H_{\text{zr}} + H_L + H_Q \tag{1.2.5}
\]

A brief introduction of all seven follows and each part of the spin Hamiltonian will be discussed in more detail in the next section. The aforementioned spin-orbit coupling which is responsible for the deviation of \( g \)-values from the free electron value is represented in the follow way:

\[
H_{L_\text{S}} = \lambda L \hat{S} + g \mu_B L \hat{B} \tag{1.2.6}
\]
Lambda is the spin-orbit coupling constant, $\mathbf{L}$ and $\mathbf{S}$ are, respectively, the orbital and spin angular momentum tensors. The typical order of $\lambda$ is about $10^2 \text{ cm}^{-1}$.

The most relevant part of the spin Hamiltonian is the electron Zeeman interaction which represents the spin energy level transition portrayed in Figure 1. The electron Zeeman Hamiltonian has the following form:

$$H_{\text{Ze}} = g_e \mu_B \mathbf{B} \cdot \mathbf{S} \quad (1.2.7)$$

It is a vector product of spin, $g$ tensor and a vector describing the external magnetic field. The electron Zeeman energy is on the order of $0.3 \text{ cm}^{-1}$ and can be highly anisotropic. On the other hand, the nuclear Zeeman interaction has three orders of magnitude lower energy and is isotropic. The nuclear Zeeman Hamiltonian is:

$$H_{\text{zn}} = -g_n \mu_N \mathbf{B} \cdot \mathbf{I} \quad (1.2.8)$$

It is analogous to the electron Zeeman interaction but is characterized by a nuclear $g$-value and is negative due to the opposite sign of the nuclear spin relative to the electron spin.

The hyperfine interaction is independent of the external magnetic field and it represents the interaction of the nuclear and the electron spins.

$$H_{\text{hf}} = \mathbf{I} \cdot \mathbf{A} \cdot \mathbf{I} \quad (1.2.9)$$

Depending on the system, the magnitude of the hyperfine interaction can range up to $1 \text{ cm}^{-1}$, although the typical range for biological systems is about $10^{-3}$ to $10^{-2} \text{ cm}^{-1}$.

Like the hyperfine, the nuclear quadrupolar interaction for nuclei where $I>\frac{1}{2}$ is external field independent and has the following form:

$$H_Q = \mathbf{Q} \cdot \mathbf{I} \quad (1.2.10)$$

Quadrupolar energies are usually in the range of $10^{-6}$ to $10^{-4} \text{ cm}^{-1}$.

The hyperfine parameter $\mathbf{A}$ and the nuclear quadrupolar parameter $\mathbf{Q}$ are of special interest for EPR spectroscopists because they define the nuclear environment of the electron spin. By elucidating these parameters the ligation and geometry of paramagnetic centers can be obtained.

The following part of the spin Hamiltonian is the electronic spin-spin interaction. It is present in systems containing more than one paramagnetic species:

$$H_J = \mathbf{S}_1 \cdot \mathbf{J} \cdot \mathbf{S}_2 \quad (1.2.11)$$

$\mathbf{J}$ tensor describes the coupling between two spins $\mathbf{S}_1$ and $\mathbf{S}_2$ couple. If the two spins are physically close to each other so that their orbitals overlap, the magnitude of their interactions could be as large as $10^3 \text{ cm}^{-1}$. The nuclear spin-spin interaction is neglected in EPR since its magnitude is about $10^{-13} \text{ cm}^{-1}$ which is well within the EPR line width for the majority of systems.
Finally, the zero-field interaction is only present in $S \geq 1$ spin systems and is a correction to the energy of individual spin states due to the spin-orbit coupling. The Hamiltonian for this interaction is expressed in the following way:

$$H_{ZF} = S_1^z \cdot D \cdot S_2$$  \hspace{1cm} (1.2.12)

Zero-filed splitting is prominent in systems with low laying excited states. The energies of this interaction largely vary and in some cases are in the order of the Zeeman energies or greater.

A detailed description of the terms that are most relevant for bioinorganic systems follows.

**The Electron Zeeman Interaction**

As mentioned above, the effective spin Hamiltonian encompasses in most cases all of the parameters that are responsible for the EPR signal represented by the effective $g$-tensor. As a consequence, the effective $g$-tensor has the following form:

$$g = g_e \begin{pmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{pmatrix} + 2\lambda \Lambda$$  \hspace{1cm} (1.2.13)

where the free electron and spin orbit coupling contributions are separated, and $\Lambda$ is a symmetric tensor with the elements

$$\Lambda_{ij} = \sum_{n \neq 0} \frac{(\psi_0 | L_i | \psi_n)(\psi_n | L_j | \psi_0)}{\varepsilon_0 - \varepsilon_n}$$  \hspace{1cm} (1.2.14)

The $\psi_0$ and $\psi_n$ are the wavefunctions of the ground state and $n^{th}$ excited state, while $\varepsilon_0$ and $\varepsilon_n$ are the energies of those states. What can be inferred from eq. 1.2.13 and 1.2.14 is that the closer in energy the exited states are to the ground state the larger the spin orbit coupling, the broader the EPR spectrum is. In most organic radicals, the gap between frontier orbitals is significantly larger than those of transition metals. For example, DFT calculations show that for a methyl radical the HOMO-LUMO gap is $\sim 50000$ cm$^{-1}$ while in Myoglobin hydroxide it is $\sim 8000$ cm$^{-1}$.

Another reason for smaller spin-orbit coupling (SOC) contributions in organic radicals is due to the relatively smaller mass of the atoms where the radical resides. This idea is expressed in the following equations:

$$\lambda = \frac{\xi}{2s}$$  \hspace{1cm} (1.2.15)  \hspace{1cm} and \hspace{1cm} $$\xi = 2\mu B Z \gamma^3$$  \hspace{1cm} (1.2.16)
where $\xi$ and $\lambda$ are SOC constants, $Z$ molecular weight, and $r$ the nuclear radius. The general trend is the spin-orbit coupling constant $\xi$ is highly dependent on the nuclear charge, heaver the atom, larger the SOC.\(^8\) Heavier atom radicals, like transition metals paramagnetic species, therefore have significantly larger SOC energies compared to organic radicals. For example, the largest principle $g$-value, $g_z$, can be expressed in both the terms of the ground and excited state energy levels and the SOC constant:\(^7\)

$$g_z = 2.0023 + \frac{8\xi}{E(0)-E(n)} \quad (1.2.17)$$

With the complexity and dependents of the $g$-tensor on various factors such as the magnetic Zeeman interaction, spin orbit coupling, and excited state energies, the three principal $g$-values themselves are merely observables and effective values. In most cases the principal $g$-values are used just as fingerprints of a paramagnetic system. To illustrate the Zeeman effect, I present the simplest CW-EPR spectrum, having only the isotropic electron Zeeman interaction ($g = 2$) with no zero-field splitting. In a magnetic field, the system will have only two energy levels associated with it. One corresponding to the spin projection $M_s = +\frac{1}{2}$, and one to the $M_s = -\frac{1}{2}$. The derivation of these energy levels are shown in Appendix A. The energy levels and the associated CW-EPR spectrum simulated in EasySpin are presented in Figure 5.\(^9\)

![Figure 1.5](image.png)

**Figure 1.5.** Simplest EPR spectrum containing isotropic $g = 2$ tensor, and its corresponding energy level diagram. The diagram indicates a single transition which results in a single peak.
The shown simulated spectrum is isotropic, but depending on the difference among the three \( g \) values an EPR spectrum can have isotropic or anisotropic (axial and rhombic symmetries). In Figure 6 are presented the examples of the shapes of symmetry of these classes of spectra as well as the spectra themselves in absorption and derivate mode. Therefore, an anisotropic EPR signal with many orientations would give orientation dependent fine structure signals.

The Nuclear Zeeman Interaction

The nuclear Zeeman interaction is a coupling of a spin active nucleus to the magnetic field. The most common nuclei used in in bioinorganic chemistry that possess a nuclear spin, values of their spins and their Larmor frequency at 3500 gauss are listed in Table 1. The Larmor frequencies can be readily correlated from the Hamiltonian. For example, a frequency from equation 1.2.8, at 3500 gauss for a proton in SI units:

\[
\nu_{Zn} = -g_n\mu_B B_0 / h = -5.586 \times 5.051 \times 10^{-27} \frac{J \cdot T}{6.626 \times 10^{-34} J s} = 14.90 \text{ MHz}.
\]

This interaction does not perturb the EPR as the selection rule for these experiments is \( \Delta M_s = 1 \), and \( \Delta M_I = 0 \). In other words, the signal from the allowed transitions immerses when the electron spin flips but the nuclear spin remains the same. To illustrate this rule, the energy diagrams and transitions with

\[
\begin{array}{ccc}
\text{ISOTROPIC} & \text{AXIAL} & \text{AXIAL} & \text{RHOMBIC} \\
\begin{array}{c}
g_1 = g_2 \equiv g_3 \\
g_1 = g_2 < g_3 \\
g_1 = g_2 > g_3 \\
g_1 \neq g_2 \neq g_3
\end{array}
\end{array}
\]

![Figure 1.6. Three basic types of symmetry governed by the principle g values and the appropriate sample EPR spectra. (adapted from reference 2)](image)

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Spin</th>
<th>Larmor freq. (MHz at 3500G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^1\text{H})</td>
<td>(\frac{1}{2})</td>
<td>14.90</td>
</tr>
<tr>
<td>(^2\text{H})</td>
<td>1</td>
<td>2.29</td>
</tr>
<tr>
<td>(^{13}\text{C})</td>
<td>(\frac{1}{2})</td>
<td>3.75</td>
</tr>
<tr>
<td>(^{14}\text{N})</td>
<td>1</td>
<td>1.08</td>
</tr>
<tr>
<td>(^{15}\text{N})</td>
<td>(\frac{1}{2})</td>
<td>1.51</td>
</tr>
<tr>
<td>(^{55}\text{Mn})</td>
<td>(\frac{5}{2})</td>
<td>3.68</td>
</tr>
<tr>
<td>(^{63}\text{Cu})</td>
<td>(\frac{3}{2})</td>
<td>3.95</td>
</tr>
<tr>
<td>(^{65}\text{Cu})</td>
<td>(\frac{3}{2})</td>
<td>4.23</td>
</tr>
<tr>
<td>(^{57}\text{Fe})</td>
<td>(\frac{1}{2})</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Table 1. Most common EPR active nuclei in Bioinorganic Chemistry, their spin values, and nuclear Larmor frequencies.
both electron and nuclear Zeeman interactions are shown in Figure 1.7.

**The Hyperfine Interaction**

The interaction between the electron and nuclear spins, the hyperfine interaction, is the primary part of the spin Hamiltonian that pulsed EPR spectroscopists are concerned with. This interaction can be used to visualize the spin density on magnetically active nuclei that surround the electron spin. Obtaining the hyperfine interaction tensors via EPR and DFT can yield great insight into the structure and function of bioinorganic systems.

It is common and convenient to parameterize this interaction in two terms: isotropic, and anisotropic. Isotropic hyperfine coupling constants arise from the Fermi contact interaction between the electron and nuclear spins. This interaction is given by:

\[
\alpha_{iso} = \frac{2\mu_B}{3\hbar} g_E g_N \mu_B |\psi_0(0)|^2
\]  

(1.2.18)

where \(|\psi_0(0)|^2\) represents the electron spin density at the nucleus. This interaction is very useful for estimating the spin density populations on neighboring nuclei.

The anisotropic part of the hyperfine interaction is in most cases a purely dipole-dipole interaction of nuclear and electronic spins through space and is represented as.
In practice the hyperfine tensor is represented as \( A = a_{iso} + T \). It is readily obtained using simulations and fits in CW, ENDOR, and ESEEM spectroscopies. This anisotropic interaction can provide insight into the spin-nucleus distance.

To understand the hyperfine interaction on its fundamental level it is particularly useful to consider the simplest case of an electronic spin \( S = \frac{1}{2} \) coupling to a nuclear spin \( I = \frac{1}{2} \). The full derivation of the energy levels of this system from basic principles can be found in Appendix A. After the set-up of the effective spin Hamiltonian, the pertinent energy levels and the corresponding EPR spectrum are shown in Figure 8. For simplicity the electron and nuclear Zeeman energies are abbreviated with \( E_z = \mu_B B_z \) and \( N_z = g_n \mu_B B_z \).

This simple model serves as a good basis for understanding more advanced techniques like ENDOR or ESEEM. More often, a single electron spin couples to many nuclei. The EPR transition energy levels thus become more complicated where each nucleus perturbs the system with its own hyperfine interaction. For example, two equivalent protons coupled to the same electron spin will give rise to three peaks, the lowest and highest energy transition peaks will have half the intensity of the middle energy transition peak due to the degeneracy of the energy levels. For nuclei such as protons, where \( I = \frac{1}{2} \), the degeneracies can be represented by the coefficients of the binomial expansion \((1+x)^{i}\) and visualized via the Pascal triangle (Figure 1.9). \(^1\) Finally, the \( g \) and the \( A \) tensor correlate via the Euler angles which are used to rotate the hyperfine into the \( g \) tensor space. \(^{11}\)
Nuclear Quadrupole Interaction

Nuclear quadrupole interaction is a term that describes an interaction of \( I \geq 1 \) nucleus with the inhomogeneous electric field caused by surrounding atoms and hence is an indication of the chemical nature of the nucleus in question. This interaction arises from the fact that high spin nuclei have a non-spherical charge distribution and thus possess a non-zero quadrupolar momentum:

\[
H_Q = \mathbf{T} \cdot \mathbf{Q} \cdot \mathbf{I} = \left( l_x l_y l_z \right) \begin{pmatrix}
Q_1 & 0 & 0 \\
0 & Q_2 & 0 \\
0 & 0 & Q_3
\end{pmatrix} \begin{pmatrix}
l_x \\
l_y \\
l_z
\end{pmatrix},
\]

where

\[
\begin{pmatrix}
Q_1 & 0 & 0 \\
0 & Q_2 & 0 \\
0 & 0 & Q_3
\end{pmatrix} = \frac{e^2 q Q}{4l(2l-1)\hbar} \begin{pmatrix}
-(1 - \eta) & 0 & 0 \\
0 & -(1 + \eta) & 0 \\
0 & 0 & 2
\end{pmatrix}
\]

Capital \( Q \) is the nucleus specific quadrupolar moment in units of barn \((10^{-28} \text{ m}^2)\), \( eq \) is the largest component of the electric field gradient, \( e \) is elementary charge, and \( \eta \) is the asymmetry parameter.

This is a purely anisotropic traceless tensor. Most commonly, the EPR selection rules are strict (i.e. there is no mixing of electron spin levels that would allow forbidden transitions) and nuclear quadrupole interaction have no effect on the EPR lineshape. However, various useful derivative EPR techniques such as ENDOR and ESEEM (see Chapter 2) could be used to extract this parameter with high accuracy.

Spin-spin coupling and Zero-field splitting

When a spin system has more than one paramagnetic entity, and those entities are weakly interacting, the zero-field splitting and spin-spin coupling interactions are taken into account. Spin-spin coupling, with its exchange coupling tensor \( J \), becomes relevant when there is significant spin orbital overlap between two spins. In these conditions, the two paramagnetic species in solid state EPR can be exchanged. The interaction is prominent when the interacting spins are closer than 15 Å. Considering a simple \( S_1 = \frac{1}{2}, S_2 = \frac{1}{2} \) system, the positive sign of \( J \) indicates a so called antiferromagnetic coupling where

[Figure 1.9. Pascal triangle where \( i \) indicates the number of equivalent nuclei coupled to the same electron spin. (adapted from reference 1)]
the triplet state is higher in energy than the singlet state. On the other hand, a negative $J$ is indicative of ferromagnetic coupling with the triplet state being the lower in energy.

The zero-field splinting interaction is a dipole-dipole interaction with a traceless tensor $\mathbf{D}$. It is an interaction analogous to the anisotropic hyperfine interaction and by characterizing this interaction in multiple spin systems, one can approximate the distance between the two interacting spin species.

1.3 Summary of Chapters

Chapter 2 is a review of theoretical and practical considerations of advanced pulse EPR, including the experimental set-up, how data is collected and processed, as well as the theory behind various techniques. In Chapter 3 Myoglobin hydroxide is introduced as a system of interest, followed by the presentation of pulsed EPR and DFT calculation data. This chapter also contains all the relevant analysis as well as experimentally and theoretically determined Fe-O distance. Appendix A is a derivation of the hyperfine interaction tensor from basic principles while Appendix B shows the Easyspin simulation used to fit experimental data.
References

Chapter 2: Pulsed EPR Theory

2.1 Technical considerations of pulse EPR

Continuous wave (CW) EPR is a very useful tool for obtaining fingerprint EPR spectra in bioinorganic chemistry as well as determining very strong hyperfine interactions of the first ligation shell to the paramagnetic metal. However, as discussed in the previous chapter, nuclear Zeeman and Quadrupole interactions are not resolved in EPR signals. Moreover, in the majority of cases, the HF splitting is well hidden within the EPR lineshape. Therefore, higher resolution spectroscopy is needed to discern these weaker interactions. This is one of the primary reasons for the development of pulsed EPR in which the response of the system is detected after excitation. Using pulsed EPR methods it is possible to resolve weak hyperfine interactions <1 MHz of nuclei in both first and second ligation shells of bioinorganic systems. To explain how pulsed ERP achieves this enhanced resolution, I will start by giving a basic experimental setup.

Figure 2.1. A scheme of principal components of a pulsed EPR spectrometer. Blue components are responsible for signal transmission, red for reception and orange for radio frequency wave emission. M.W - the microwave source, PFU – pulse forming unit, M.W. Amp. – microwave amplifier, H.P.Att. – high power attenuator, R.F. – radio frequency source, R.F. Amp. - Radio frequency amplifier, LNA – low-noise amplifier
Figure 2.1 depicts simplified principal components of a pulsed EPR spectrometer. The microwaves are first generated by a high quality microwave source. Then the irradiation is gated by a computer controlled pulse forming unit to create the desired sequence of pulses. The signal is then amplified to very high powers, for X-band frequencies (9.5 GHz) the typical output power levels are about ~1kW as achieved by a traveling wave tube (TWT) amplifier. If necessary, the generated MW pulses are attenuated, and sent into the MW resonator with a latched in a magnetic field. The levels of power that we detect is in units of pW, thus, the detection instrumentation is very sensitive to high powers. To avoid the possible damage, during the duration of the MW pulses the detection is “switched off”. Then, the response of the system is converted from MW signal to DC. Then, the resulting signal will be amplified, phase corrected and sent to the computer for processing.

An important consideration of pulse EPR is the pulse bandwidth (Figure 2.2) defined as the range of frequencies between the first left and right zero crossing of the Fourier transform of a pulse of length $t_p$.\(^{13}\)

$$\text{BW} = \frac{2}{t_p} \quad (2.1.1)$$

*Figure 2.2* Comparison of CW vs pulsed EPR bandwidths. In CW EPR the bandwidth is infinitely small, while in pulsed EPR it is inversely proportional to the pulse length.
To obtain a basic idea about how spin systems respond to a pulsed perturbation, a classical representation of a spin system, with magnetization vector illustrations is suitable.\textsuperscript{13}

### 2.2 Classical description of pulsed EPR

Magnetic dipoles of paramagnetic species in an external magnetic field precess around the $\mathbf{B}_0$, the laboratory magnetic field vector with the net magnetization $\mathbf{M}$ with the frequency\textsuperscript{5}

$$\omega_0 = \frac{g\mu_B B_0}{h} \quad \text{(2.2.1)}$$

The microwave generated magnetic field $\mathbf{B}_1$ induces an additional nutation motion on the net magnetization $\mathbf{M}$, tipping it from the z-axis toward the xy plane. The effects of a microwave pulse on the net magnetization are presented in Figure 2.3.\textsuperscript{13} The tip angle $\alpha$, away from the z-axis is proportional to the pulse length $t_p$ and the strength of the magnetic field $B_1$ generated by the microwave power:

$$\alpha = -\gamma_e |\mathbf{B}_1| t_p \quad \text{(2.1.2)}$$

A $\pi/2$ pulse from the direction of the x-axis will nutate the magnetization onto the $-y$ axis. This effect of $\mathbf{B}_1$ on the net magnetization is hard to envision in the laboratory Cartesian coordinate framework. Instead, by rotating the coordinate system with an angular velocity $\omega_0$, we can make one of the components of $\mathbf{B}_1$ appear stationary. This way, for simplicity sake, we have effectively “switched off” the $B_0$ induced precession. All the further discussion of the magnetization vector will be placed in the rotating frame.
Free Induction Decay

The simplest pulsed EPR experiment is called the free induction decay (FID). A microwave pulse that is long enough to allow the net magnetization to rotate from $z$ to $y$ of the rotating frame (i.e. $\pi/2$ pulse) thus inducing a current and a detectable voltage that can be transformed into a signal. Due to the inhomogeneity of the external and internal magnetic fields, the electron spins of each individual paramagnetic species will precess at a different frequency in the $xy$ plane $\omega - \omega_0$. As a consequence the net magnetization decays rapidly. To illustrate the FID effect, a time dependence of the magnetization vector $M_y$ is presented in figure 2.4. Unfortunately, there is a severe limitation to performing pulse EPR FID measurements. After every pulse, due to cavity-ringing, there is an associated delay of detection about 100ns long. This delay is called “dead time”, $t_D$ and during this time, the microwave power in the cavity is so high that the signal cannot be detected. With this limitation in mind, a very small portion, if any, of the FID can be detected. To overcome this limitation, the spin-echo technique is used.

\[ \begin{align*}
M_y &= 1 \\
M_y &= 0 \\
M_y &= 1 \\
M_y &= 0
\end{align*} \]
**Two-pulse echo**

The simplest spin-echo technique was first discovered by Hahn for nuclear spins using a $\pi/2 - \tau - \pi/2$ pulse sequence and was later perfected by Carr and Purcell to a $\pi/2 - \tau - \pi$.\(^{15,16}\) In the latter sequence, the first $\pi/2$ pulse puts the magnetization vector on the $y$ axis and the electrons spins “fan out,” as described above. After a time $\tau$, a $\pi$ pulse is applied thus reversing the precessing direction and position of each individual spin packet, while their frequencies remain the same. After a time $\tau$ following the second pulse, all magnetization vectors are aligned back along the $-y$ direction thus building up a “spin echo.” The pulse sequence, as well as a scheme of the formation of the two pulse spin echo in the rotating frame is shown in Figure 2.5.

![Figure 2.5. Two-pulse spin echo sequence and its spin magnetization vector scheme](image)

This basic pulse sequence allows us to get away from the “dead time,” the acquisition delay time after every pulse and obtain an entire spin echo signal. The amplitude of the echo is a function of $\tau$ during which the magnetization is in the $xy$ plane. As $\tau$ increases, the intensity of the echo decays due to the so called phase memory time $T_m$ that corresponds to a time during which the individual spin packets keep their phase during their precession in the $xy$ plane. This loss of “phase memory” in mainly due to weak interactions between the electronic spins in the ensemble. Unfortunately, in practice $T_m$ can be very short giving very poor spectral resolution in such experiments. To overcome this limitation, the three-pulse stimulated echo pulse sequence was developed.\(^{17}\)
**Three-pulse echo**

The three-pulse or the stimulated echo is generated via a $\pi/2 - \tau - \pi/2 - T - \pi/2$ pulse sequence. Analogous to the two-pulse, the magnetization after the first pulse is precessing in the $xy$ plane. After a time $\tau$, a component $\delta M$ of the net transverse magnetization $M$ makes an angle $\theta = (\omega - \omega_0) \tau$ with respect to the $y$ axis, where $\omega_0$ is the MW pulse frequency and $\omega$ is the precession frequency of the component $\delta M$ in the laboratory field axis. The second $\pi/2$ pulse after a time $\tau$ brings $\delta M$ into the $xz$ plane retaining the angle $\theta$ now between $\delta M$ and the $-z$ axis. At this moment, $\delta M$ can be projected onto two components a transverse $\delta M_x$ and longitudinal $\delta M_z$. The $\delta M_x$ component makes a so-called “eight ball” spin echo at a $\pi/2 - \tau - \pi/2 - \tau$. The longitudinal component $\delta M_z$ now decays on the $z$-axis. Finally, the third MW pulse in the sequence torques $\delta M_z$ on the $-y$ axis converting it to $\delta M_y$. After a time $\tau$ the stimulated echo is formed (Figure 2.6). The advantage of the three- vs two-pulse echo lies in the fact that the second $\pi/2$ pulse serves to store the precession frequency offsets $(\omega - \omega_0)$ on the $z$-axis, that is in the spin-lattice relaxation time $T_1$. This time represents the time required for the net spin magnetization to return to equilibrium, to the $z$ axis, following a $\pi/2$ pulse. In most biologically relevant cases this time is temperature dependent, increasing at lower temperatures, and typically much longer than $T_2$. By lowering the temperature we can allow longer delay times $T$ and thus improve spectral resolution. The three-pulse sequence, apart from the stimulated ESE has three additional primary echoes arising from each pulse pair. In most experiments we are concerned only with the stimulated one. If $\tau$ or $T$ are changing, any unwanted echoes can overlap with stimulated one thus creating artifacts. Phase cycling is used to eliminate these signals. In this procedure, the pulse sequence is repeated several times by altering pulse phases and summing up the result. In turn, this phase change alters the phase of the ESE signals with respect to detection. Thus, by applying a proper combination of phases
one can eliminate the unwanted echoes and FIDs. For example, in the stimulated three-pulse echo: addition of the signals arising from the pulses with phases (+x +x +x) and (-x -x +x) and the subtraction of signals that come (-x +x +x) and (+x -x +x) pulse phases results in the removal of unwanted echoes and amplification of the echo of interest (Figure 2.7).\textsuperscript{19}

\textit{Four-pulse echo}

The four-pulse spin echo sequence and the corresponding formation of the stimulated echo are similar to the aforementioned three-pulse spin echo. In this case a $\pi$ pulse is inserted between the second and the third pulse resulting in an overall pulse sequence $\pi/2 - \tau - \pi/2 - T_1 - \pi - T_2 - \pi/2$ with the inverted echo forming after a time $\tau$ after the last pulse (Figure 2.8).
Following the description of the three-pulse sequence we focus on the time point after the second π/2 pulse with the prominent $\delta M_z$ component on the $-z$ axis. By applying the π pulse after a time $T_1$ following the second pulse, the $\delta M_z$ is transferred on the z axis. With an application of the last π/2 pulse, this magnetization component goes to the xy plane converging onto the y axis and resulting in an inverted echo. The decay rate of the amplitude of the four-pulse spin echo is determined by both $T_M$ and spin lattice relaxation $T_1$ because the magnetization is stored both in the xy plane for time $\tau$ and on the z-axis for a time $T_1+T_2$. To understand what information we can obtain from these experiments we will need to introduce an approach that greatly simplifies the description of pulse EPR experiments called the density matrix formalism.

2.3. Density Matrix formalism

This approach has been extensively discussed in the literature, while we will just cover the basic principles here.\(^5\)

Any quantum mechanical system is described by a set of orthogonal functions:

$$|\psi_1> \ldots |\psi_n> \quad (2.3.1)$$

In a case of a spin system we can specify that a given state can be expressed in the basis of spin states:

$$|\psi> = \sum_j (c_{\alpha j}|\alpha_j> \ldots \pm c_{\beta j}|\beta_j>) \quad (2.3.2)$$

where $j$ counts the number of spins in the system and the complex coefficients satisfy the normalization condition

$$\sum_j |c_{\alpha j}|^2 + |c_{\beta j}|^2 = 1 \quad (2.3.3)$$
A convenient way of describing the evolution of a spin system is via evolution of its density matrix

\[
\sigma = \begin{pmatrix}
|c_{\alpha_1}|^2 & \cdots & |c_{\alpha_n} c_{\beta_n^*}|^2 \\
\vdots & \ddots & \vdots \\
|c_{\alpha_1} c_{\beta_1^*}|^2 & \cdots & |c_{\beta_n}|^2
\end{pmatrix}
\] (2.3.4)

This approach is called the density matrix formalism in which the expectation value of any operator is simply a trace of the product of the density matrix with this operator in question such that:

\[
\langle A \rangle = \text{tr}(A \sigma)
\] (2.3.5)

For example, a magnetization vector along the z axis,

\[
M_z = \langle S_z \rangle = \text{tr}(S_z \sigma)
\] (2.3.6)

If the evolution of a spin system in time is described by the Hamiltonian \(H(t)\) so that

\[
\frac{d|\psi\rangle}{dt} = -i H(t) |\psi\rangle
\] (2.3.7)

then, using the density matrix representation one can write the time dependence of the matrix \(\sigma(t)\)

\[
\frac{d\sigma(t)}{dt} = -i[H(t), \sigma(t)]
\] (2.3.8)

Which is the Liouville-von Neumann equation that is a key equation in describing the general behavior of pulse experiments. In the case when the Hamiltonian \(H\) is time-independent, the formal integration of eq. 2.3.8 yields

\[
\sigma(t) = e^{-iHt} \sigma(0) e^{iHt}
\] (2.3.9)

The exponential operator responsible for the propagation of the spin system in time is called the propagator and is given as:

\[
U(t) = e^{-iHt}
\] (2.3.10)

For a pulse experiment, evolution of the density matrix can be represented as a sequential act of individual perturbations be a MW pulse or a waiting period. Then the density operator at the time of detection is
\[ \sigma_{\text{det}} = U_n \cdots U_2 U_1 \sigma(0) U_1^\dagger U_2^\dagger \cdots U_n^\dagger \quad (2.3.11) \]

Therefore the electron spin echo would be proportional to the calculated magnetization

\[ M_y = \langle S_y \rangle = \text{Tr} (\sigma_{\text{det}} S_y) \quad (2.3.12) \]

Hence, any pulse sequence can be calculated from first principles by using simple multiplication of matrices.

### 2.4. Electron Nuclear Double Resonance – ENDOR

ENDOR is a double resonance technique in which the excitation of an NMR transition is detected via the changes in the electron spin echo.\(^{20}\) There are two main pulse sequences that are commonly used in pulsed EPR experiments, Davies and Mims ENDOR. Mims ENDOR is a technique for determining weakly coupled nuclei.\(^{21}\) In addition, this technique suffers from the “blind-spot” effects similarly to the three-pulse ESEEM (see section 2.5). This method is not appropriate for the determination of the strongly coupled \(^1\)H nucleus and therefore we focus only on the theory of the Davies sequence.

**Davies ENDOR**

Davies ENDOR sequence consists of three microwave pulses with an addition of a radio frequency pulse between the first and the second MW pulses (Figure 2.9.).\(^{22}\)

![Figure 2.9. Davies ENDOR pulse sequence and its electron spin magnetization vector scheme](image)

The length of the RF pulse strongly depends on the experimental set up. For a commercial X-band spectrometer from Bruker, the typical values of \( t_{\text{RF}} \) for proton transitions are about 15 \( \mu s \).\(^{2} \) All the pulses in Davies ENDOR sequence are stationary while the signal is recorded as a function of RF
frequency. The basic theory of Davies ENDOR is explained using the $S=\frac{1}{2}$, $I=\frac{1}{2}$, model system (Figure 2.10).\(^{13}\) The first MW π pulse inverts the populations of the energy levels 1 and 3. The inverted echo intensity is dependent on that population difference $\Delta$. When the frequency of the following RF π pulse matches $\omega_\alpha$ the populations of the energy levels 1 and 2 invert thus decreasing the inverted echo intensity. Similarly, echo decrease will result when RF π pulse matches $\omega_\beta$. As a result, two peaks corresponding to $\omega_\alpha$ and $\omega_\beta$ are observed. From the energy levels portrayed in Appendix A, we can isolate two cases, one when the nuclear Larmor frequency $\nu_L$ is greater than half the hyperfine coupling constant $\frac{A}{2}$ and one when it is smaller. In the first case the ENDOR signal is a doublet centered on $\nu_L$ and split by $A$, and in the second case the ENDOR doublet is centered around $\frac{A}{2}$ and split by $2\nu_L$. One important consideration for Davies ENDOR is the bandwidth of the first pulse in the sequence, the inversion pulse. If the bandwidth is large enough to excite both 1-3 and 2-4 electron spin transition, the RF pulse will have no effect on the populations yielding no ENDOR response. This effect (see Chapter 3) can also be used to suppress signals coming from unwanted weakly coupled nuclei.

2.5. Basic principles and the semiclassical representation of Electron Spin Echo Envelope Modulation (ESEEM)

All ESEEM experiments are carried out by measuring the integral of the transient electron spin echo signal as a function of the time between pulses. With a large enough MW pulse bandwidth and when the allowed and semi-forbidden transitions are simultaneously excited the time dependance of the signals will be modulated.\(^{23}\) These time domain modulations correspond to frequencies of nuclear transitions related to the electron spin and thus contain information about various interactions related to electron and nuclear spins.
Two-pulse ESEEM

In the two-pulse ESEEM experiment, the echo intensity is recorded as a function of $\tau$, the time interval between the two pulses. A scheme of the pulse sequence and the resulting echo modulation is presented in Figure 2.11.

Let us consider a simple system consisting of a single unpaired electron $S = \frac{1}{2}$, coupled to an $I = \frac{1}{2}$ nucleus.
described in Appendix A. The four energy levels portray six transitions of which two are allowed electron spin transitions \( \omega_{13} \) a \( \omega_{24} \), two are forbidden spin electron transitions \( \omega_{23} \) \( \omega_{14} \), and two are nuclear transitions \( \omega_a \) \( \omega_b \) (Figure 2.12). If the MW pulse bandwidth is wide enough, all four electron transitions \( \Delta M = 1 \) are excited simultaneously. ¹⁸ Let us consider a somewhat special case when only the \( \omega_{13} \) and \( \omega_{23} \) transitions are accessible. After the initial \( \pi/2 \) pulse from the x direction both 1-3 and 2-3 transition vectors point along the y-axis of the rotating frame. If the transition probabilities 1-3 and 2-3 are defined as \( |T_{13}|^2 \) and \( |T_{23}|^2 \) the magnitudes of the \( \omega_{13} \) and \( \omega_{23} \) vectors correspond to those transition probabilities. Immediately after the first pulse, the vector \( \omega_{23} \) starts precessing with a frequency \( \omega_{13} - \omega_{23} = \omega_a \) while \( \omega_{13} \) remains stationary in the rotating frame. After a time \( \tau \), the \( \omega_{23} \) vector makes an angle of \( \omega_a \tau \) with the y-axis, at which point the \( \pi \) pulse is applied thus flipping the two vectors \( \omega_{13} \) and \( \omega_{23} \) to the \(-y\) direction. In addition, the \( \pi \) pulse transfers some of the energy level populations in such a way that it creates two new vectors \( \omega_{13}' \) and \( \omega_{23}' \) where \( \omega_{13}' = \omega_{23} \) and \( \omega_{13} = \omega_{23}' \). ¹⁸ The magnitude of the vectors \( \omega_{13}' \) and \( \omega_{23}' \) correspond to the product of the transition probabilities \( |T_{13}|^2 \) \( |T_{23}|^2 \) and \( |T_{13}|^2 \), respectively. During the second evolution time, the \( \omega_{13} \) and \( \omega_{13}' \) remain stationary while \( \omega_{23} \) and \( \omega_{23}' \) precess with the angular velocity \( \omega_a \). At the moment of the spin echo, \( \tau \) after the \( \pi \) pulse, the \( \omega_{13} \) and \( \omega_{23} \) vectors lay on the \(-y\) axis while \( \omega_{13}' \) and \( \omega_{23}' \) make an angle of \( \omega_a \tau \). The resulting echo signal produced by the 1-3 and 2-3 transitions is given by: ²⁴

\[
E(\omega_{13}, \omega_{23}, \tau) = |T_{13}|^4 + |T_{23}|^4 + 2 |T_{13}|^2 |T_{23}|^2 \cos \omega_a \tau \tag{2.5.1}
\]

The total \( \delta M_y \) magnitude at time of the spin echo depends both on \( \tau \) and \( \omega_a \). As a result, the echo intensity is modulated as a function of \( \tau \) and contains the desired frequency \( \omega_a \) which is a combination of the nuclear Zeeman and hyperfine interaction. This example is focused on the correlation of the 1-3 and 2-3 energy level transitions. Analogously, this process can be repeated for all pairs of electron spin transitions. Because \( |T_{13}|^2 = |T_{24}|^2 = |T_a|^2 \) \( |T_{14}|^2 = |T_{23}|^2 = |T_b|^2 \) the complete normalized amplitude of the two-pulse spin echo is expressed as: ²⁴

\[
E(\tau) = 1 - \frac{k}{4} [2 - 2\cos(\omega_a \tau) - 2\cos(\omega_b \tau) + \cos(\omega_\alpha \tau) + \cos(\omega_\beta \tau)] \tag{2.5.2}
\]

where \( k = 4|T_a|^2 |T_b|^2 \), \( \omega_\alpha = \omega_a - \omega_b \) and \( \omega_\beta = \omega_a + \omega_b \). Therefore, the two-pulse ESEEM echo amplitude is dependent on both the fundamental nuclear frequencies \( \omega_a \), \( \omega_b \) as well as their combinations \( \omega_\alpha \), \( \omega_\beta \), while the parameter \( k \) defines the modulation depth.⁵

\[
k = \left( \frac{B\omega_1}{\omega_\alpha \omega_\beta} \right)^2 \tag{2.5.3}
\]
The k parameter is dependent on $\omega_\alpha$, $\omega_\beta$, $\omega_1$, the nuclear Larmor frequency, and the anisotropic part of the hyperfine interaction B. Therefore, if the hyperfine interaction is purely isotropic, there is no ESEEM effect. Sample simulated two-pulse ESEEM spectra are presented in Figure 2.13.

![Figure 2.13. Simulated two-pulse ESEEM spectra of the $^{14}$N nucleus in time and frequency domain](image)

**Three-pulse ESEEM**

In this experiment, the stimulated echo is measured as a function of the time delay, T, between the second and the third $\pi/2$ pulse (Figure 2.6). Like the two-pulse ESEEM, the first $\pi/2$ pulse generates the electron coherences. Then, the second $\pi/2$ pulse stores the nuclear coherences onto the $-z$ axis where they develop during the delay time T. The final $\pi/2$ pulse transfers these coherences onto the $xy$ plane where the echo is formed and detected. For our $S=\frac{1}{2}$, $I=\frac{1}{2}$ spin system the echo amplitude formula is simplified to:

$$E(t,T) = 1 - \frac{k}{4} \left( [1 - \cos(\omega_\alpha \tau)] [1 - \cos(\omega_\beta (\tau+T))] + [1 - \cos(\omega_\beta \tau)] [1 - \cos(\omega_\alpha (\tau+T))] \right)$$

(2.5.4)

The modulation amplitudes of nuclear frequencies in this case depend on both the modulation depth parameter $k$ and the time internal $\tau$ between the first and second pulses. Thus, there is a $\tau$ suppression effect that distorts the line-shapes of the three-pulse ESEEM frequency domain peaks. The signal also suffers from the so called “blind-spot” effect. As it can be inferred from eq. (2.5.4) when $\tau = \frac{2n\pi}{\omega_\alpha}$, where $n = 1,2,3 \ldots$, the amplitude of the $\omega_\beta$ modulation component is 0 and vice versa. Although this effect
prevents the observation of the entire nuclear line-shapes it can be used to emphasize different parts of the ESEEM spectra. Sample simulated spectra are shown in Figure 2.14.

$$E(\tau, T_1, T_2) = \frac{1}{2} \left[ E^{a\beta}(\tau, T_1, T_2) + E^{\beta a}(\tau, T_1, T_2) \right]$$

where

$$E^{a\beta}(\tau, T_1, T_2) = 1 - \frac{k}{2} \left[ \frac{C_0}{2} + C_4 \left( \cos(\omega_{12}(T_1 + \frac{T}{2})) + C_\beta \cos(\omega_{34}(T_2 + \frac{T}{2})) \right) \right.$$ 

$$+ C_c \left( \cos^2 \eta \cos(\omega_{12}T_1 + \omega_{34}T_2 + \omega_+ \frac{T}{2}) + \sin^2 \eta \cos(\omega_{12}T_1 - \omega_{34}T_2 - \omega_+ \frac{T}{2}) \right) \right]$$

$$E^{\beta a}(\tau, T_1, T_2) = 1 - \frac{k}{2} \left[ \frac{C_0}{2} + C_4 \left( \cos(\omega_{12}(T_2 + \frac{T}{2})) + C_\beta \cos(\omega_{34}(T_1 + \frac{T}{2})) \right) \right.$$ 

$$+ C_c \left( \cos^2 \eta \cos(\omega_{12}T_2 + \omega_{34}T_1 + \omega_+ \frac{T}{2}) + \sin^2 \eta \cos(\omega_{12}T_2 - \omega_{34}T_1 - \omega_+ \frac{T}{2}) \right) \right]$$

**HYSCORE-Hyperfine Sublevel Correlation Spectroscopy**

The HYSCORE experiment is performed via the four-pulse echo sequence as a function of the time delays $T_1$ and $T_2$ (Figure 2.8). The third $\pi$ pulse is centered between the $2^{\text{nd}}$ and the $4^{\text{th}}$ $\pi/2$ pulses. The expression for the echo amplitude for the $S=\frac{1}{2}$, $I=\frac{1}{2}$ spin system is:

$$E(\tau, T_1, T_2) = \frac{1}{2} \left[ E^{a\beta}(\tau, T_1, T_2) + E^{\beta a}(\tau, T_1, T_2) \right]$$
The advantage of the HYSCORE technique is the increase in resolution due to the fact that the delay before and after the $\pi$ pulse are changed independently. This way, the signals arising from different nuclei are usually well separated. Also, in HYSCORE the echo decays as the spin-lattice relaxation $T_1$ time allowing for longer detection times. Like the three-pulse ESEEM, HYSCORE suffers from the interference of unwanted echoes. A four step phase cycle $+ (+x +x +x +x)$, $+(-x -x +x +x)$, $+(+x +x -x +x)$, $+(-x -x -x +x)$ is used to remove those unwanted echoes. Sample simulated spectra are shown in Figure 2.15.

\begin{align}
C_0 &= 3 - \cos(\omega_{12} \tau) - \cos(\omega_{34} \tau) - \sin^2 \eta \cos(\omega_+ \tau) - \cos^2 \eta \cos(\omega_- \tau) \\
C_\alpha &= \cos^2 \eta \cos \left( \left( \omega_{34} - \frac{\omega_{12}}{2} \right) \tau \right) + \sin^2 \eta \cos \left( \left( \omega_{34} + \frac{\omega_{12}}{2} \right) \tau \right) - \cos \left( \frac{\omega_{12} \tau}{2} \right) \\
C_\beta &= \cos^2 \eta \cos \left( \left( \omega_{12} - \frac{\omega_{34}}{2} \right) \tau \right) + \sin^2 \eta \cos \left( \left( \omega_{12} + \frac{\omega_{34}}{2} \right) \tau \right) - \cos \left( \frac{\omega_{34} \tau}{2} \right) \\
C_c &= -2 \sin \left( \frac{\omega_{12} \tau}{2} \right) \sin \left( \frac{\omega_{34} \tau}{2} \right). \quad (2.5.5)
\end{align}

Figure 2.15. Simulated HYSCORE spectra of the $^{14}$N nucleus in time and frequency domain
References

Chapter 3:

Advanced Pulse EPR techniques applied to the hydroxyl proton of Myoglobin Hydroxide.

3.1 Myoglobin Hydroxide, an introduction

Myoglobin (Mb) is an oxygen storage protein found in muscle tissue of most mammals. This 154 amino acid long globular protein utilizes the heme cofactor to bind and store oxygen, releasing into the blood stream upon muscle injury. The Fe-cofactor is a heme protoporphyrin IX ligated by an axial histidine and a water molecule. Above pH 9, the six coordinate aqua adduct deprotonates to Myoglobin Hydroxide MbOH. The electron spin in this system lies in one of the $t_{2g}$ orbitals on the low-spin ferric iron (Figure 3.1). It is surrounded by several spin active nuclei and has been studied both via EPR and DFT methods previously. However, the hyperfine tensor of the hydroxyl proton has not been determined yet. The goal of this project is to elucidate this hyperfine tensor via a range of pulse EPR techniques, such as ESEEM, ENDOR, HYSCORE as well as Density Functional Theory (DFT). The obtained HF coupling constant can be then related to iron-oxygen distances via the point-dipole approximation. In turn, these distances can be compared with ones obtained experimentally allowing us to estimate the accuracy of various magnetic resonance techniques.

The point-dipole approximation assumes that the electron spin is located far enough from a nucleus that the character of the spin distribution can be neglected. Then, the magnitude of the anisotropic hyperfine constants can be used to estimate distances between nuclear and electron spin as shown by the following formula:

$$T_\perp = \frac{g_n g_e V_{Q MB}}{\hbar r^2 \varepsilon_0} \quad (3.1.1)$$
where $T_\perp$ is the non-unique part of dipolar tensor of the hyperfine interaction, $g_e, g_n$ are the electron and nuclear $g$ values, $\mu_B, \mu_{NB}$ their respective Bohr magnetons, $\epsilon_0$ the permeability of vacuum, and $r$ the distance between the nucleus in question and the center of mass of the spin density. This approximation has a lower limit (distance greater than 2.2 Å) to the distance determination but has been only utilized before on a different heme system$^{31}$. By evaluating the hyperfine tensor via several EPR techniques and comparing the results with more standard distance determination methods we will be both able to evaluate each technique and assess its potential in more intriguing systems such as Cytochrome P450.

3.2 Results and Discussion

**Pulsed, Field Swept EPR of MbOH**

The basic EPR experiment described in Chapter 1 is the continuous wave EPR field swept method. Similarly in pulsed EPR, a dependence of the electron spin echo could be recorded as a function of the magnetic field resulting in an absorption-like EPR spectrum. The established values of the $g$ tensor of MbOH $g = [2.6 2.16 1.83]^{27}$ were reproduced well, however, the shape of the spectrum was highly dependent on the method (Figure 3.2). The simulated spectrum resembles closely the integral of the standard CW spectrum of MbOH (not shown). The spectrum closest to the simulation is the FID detected signal. The two-pulse field swept ESE spectra suffer from field dependent electron spin echo modulation. As described in Chapter 2, the two-pulse echo is subject to the ESEEM effect. In this case the ESEEM effect is both $\tau$ and field dependent, resulting in distortions. This deviation is very prominent around $g = 2.3$ where the signal is almost completely suppressed. Based on the data published earlier these ESEEM modulations most likely correspond to strongly coupled nitrogen of the porphyrin ring.$^{28}$

![Figure 3.2. Field swept EPR spectra of MbOH at 10K. a) In blue dotted line the simulation, b) in red the FID detected signal, c) in black the two pulse echo detection, $\tau = 140$ns, and d) in green the two pulse echo detection, $\tau = 300$ns](image)
Davies ENDOR of MbOH

Davies ENDOR is a well-established technique for determination of strongly coupled protons. As discussed in Chapter 2, choosing a relatively short inversion pulse allows the suppression of the signals of weakly coupled “matrix” protons leaving only the hydroxyl $^1$H signal with a coupling of 10MHz at 3678G (Figure 3.3). In order to confirm this signal assignment and subtract baseline effects we have performed the same experiment with a Myoglobin sample that was buffer exchanged into D$_2$O. The solvent exposed water in the heme is readily exchangeable and thus by using the D$_2$O buffer we can easily replace the hydroxyl proton with a deuteron. The deuterium nucleus has a gyromagnetic ratio that is 6.15 times smaller than that of a proton and since the hyperfine interaction scales with this ratio, the signals of the hydroxyl deuteron scales down to the lower frequency region. Thus, if our assignment of the strongly coupled proton to the hydroxyl ligand is correct, we should see a disappearance of this signal in the proton region of the ENDOR spectrum and the appearance of a smaller doublet centered around 2.2 MHz. The experiment confirmed the assignment as can be clearly seen in (Figure 3.3). In order to fully determine the principle HF coupling constants we have performed the experiment at five different field positions and simulated the data using EasySpin (Figure 3.4). The hyperfine parameters

![Figure 3.3. Davies ENDOR spectra of MbOH at 4.2K at 3678G. In red MbOH, in blue MbOD and in black the difference spectrum.](image)
and Euler angles obtained were $^1H A_{\text{dip}} = [5.5\ 6.5\ -12] \pm 0.25 \text{ MHz}$ and $A_{\text{iso}} = 5 \pm 0.25 \text{ MHz}$, and $E_{\text{xyz}} = [0\ 55\ 155] \pm 2.5 \text{ degrees}$.

Figure 3.4. Davies ENDOR spectra of MbOH at 4.2K, at 2730G 3000G 3226G 3417G 3678G centered at $^1H$ Larmor frequency. In red are the experimental data and in blue the simulation.
Two-pulse ESEEM of MbOH

The echo of two-pulse ESEEM technique described in Chapter 2, is dependent on the phase memory time $T_m$. In the case of MbOH, this echo decay relaxation time is very short resulting in complete disappearance of the echo signal already at 800ns (Figure 3.5). In addition to the short $T_m$ a single very large modulation at ~200ns appears. As a result the Fourier transform data of the two-pulse ESEEM showed no clear strongly coupled $^1H$ peaks rendering the experiment unsuccessful. The modulation of about 5 MHz frequency that is visible in the Figure 3.5 is likely due to the $^{14}N$ nuclei. However, as the spectral resolution in the this experiment is very poor, no definite assignment could be obtained even for this strong modulation.

Figure 3.5. The time domain, two-pulse ESEEM spectrum of MbOH at 10K, at 3678G.
Three-pulse ESEEM of MbOH

Unlike the two-pulse, the three-pulse ESEEM does not decay with $T_m$, but rather with the longitudinal relaxation $T_1$ (see Chapter 2) which is much longer in the case of MBOH. Therefore we were able to obtain significantly higher spectral resolution (Figure 3.6).

![Figure 3.6. The time domain, three-pulse ESEEM spectrum of MbOH at 10K, at 2898.](image)

Even though the spectra are dominated by the $^{14}$N modulations, at certain field position and $\tau$ values the strongly coupled hydroxyl proton is clearly visible. The Fourier transform of these data and the appropriate simulations of the three-pulse ESEEM are presented in Figure 3.7.

![Figure 3.7. The FT three-pulse ESEEM spectra of MbOH at 10K. On left, a full spectrum indicating strong $^{14}$N signals. On right, the zoomed-in region of the hydroxyl proton data (in red) at 2898G and 3112G, and appropriate simulations (in blue).](image)
Unfortunately, the low frequency hydroxyl proton peak is not visible due to the signal overlap with the dominating nitrogen signals. Nevertheless, the simulations indicate hyperfine parameters and Euler angles of $^{1}\text{H} A_{\text{dip}} = [5.4 \ 5.4 \ -10.8] \pm 0.5 \text{ MHz}$ and $A_{\text{iso}} = 5.3 \pm 0.5 \text{ MHz}$, and $E = [0 \ 50 \ 155] \pm 2.5 \text{ degrees}$. Due to the distorted lineshapes of three-pulse ESEEM data and the absence of the low frequency peaks, the fits have much lower quality compared to the data of Davies ENDOR which in turn results in higher uncertainty range for the obtained couplings. Still, the results are in reasonable agreement indicating that the three-pulse ESEEM method is a valuable technique.

**HYSCORE of MbOH**

Compared to three-pulse ESEEM, the HYSCORE technique requires significantly longer acquisition times, with the benefit of increased resolution. As a two dimensional technique, with HYSCORE we are able to separate the signals of different nuclei. In the case of MbOH, the nitrogen signals are clearly separated from the desired proton signals (Figure 3.8) allowing unperturbed observation of the proton ridges. Therefore, the simulation of such two dimensional spectra are much more accurate than the one dimensional ones. The simulated $^{1}\text{H}$ hyperfine tensor and Euler angles are: $^{1}\text{H} A_{\text{dip}} = [5.3 \ 5.9 \ -11.2] \pm 0.375 \text{ MHz}$ and $A_{\text{iso}} = 4.1 \pm 0.375 \text{ MHz}$, and $E = [0 \ 20 \ 190] \pm 2.5 \text{ degrees}$.

![Figure 3.8. The FT HYSCORE spectra of MbOH at 10K. A) A Full spectrum indicating both $^{14}\text{N}$ and $^{1}\text{H}$ signals. B) and C) are HYSCORE data at 3757G and 3150G respectively, zoomed in on the $^{1}\text{H}$ proton region while D) and E) are the appropriate simulations](image)
Compared to the three-pulse ESEEM experiments, the HYSCORE data analysis results in more anisotropic HF coupling, which is in fact closer to the values obtained via ENDOR.

**DFT calculations of MbOH hydroxyl proton hyperfine tensor**

Density functional theory has been widely used to calculate hyperfine interactions of bioinorganic systems.\(^\text{33}\) The hyperfine tensor is calculated as \( A = a_{\text{iso}} + T \), via the spin density in the following manner for the isotropic coupling constant:\(^\text{34}\)

\[
a_{\text{iso}} = \frac{4\pi}{3} g_n g_e \mu_B H_{NB} < S_z >^{-1} \sum_{\mu,\nu} P_{\mu,\nu}^{\alpha=\beta} < \psi_\mu(r_{kN}) | \delta^2 | \psi_\nu(r_{kN}) >
\]

(3.2.1)

and the components of the anisotropic matrix for a nucelous \( N \):\(^\text{35}\)

\[
T_{ij}^{(N)} = \frac{1}{2} g_n g_e \mu_B H_{NB} < S_z >^{-1} \sum_{\mu,\nu} P_{\mu,\nu}^{\alpha=\beta} < \psi_\mu(r_{kN}) | r_{kN}^{-5} (r_{kN}^2 \delta_{ij} - 3 r_{kN} r_{kN}^2 \delta_{ij}) | \psi_\nu(r_{kN}) >
\]

(3.2.2)

where \( i, j = x, y, z \) beside the standard magnetic parameters, the spin density at the nucleus is obtained from the corresponding atomic orbitals, and \( P_{\mu,\nu}^{\alpha=\beta} \) is an element of the unpaired spin density matrix. For the model of MbOH shown in Figure 3.1, DFT calculations using the B3LYP functional and the 6-311g basis set result in a spin density distribution depicted in Figure 3.9.

---

**Figure 3.9.** Calculated spin density of MbOH. The yellow isosurface represents the \( |\alpha> \) spin density and the red isosurface the \( |\beta> \) spin density.
Although the majority of the spin density is on the iron $d_{yz}$ orbital, a significant portion of the density is located around the oxygen. Resulting hyperfine couplings of the hydroxyl proton are greatly overestimated with $^1H A_{\text{dip}} = [-11 - 8 19]$ and $A_{\text{iso}} = 10$ MHz, with the magnitude of the largest coupling $A_{\text{max}}$ (DFT) almost as large as $2A_{\text{max}}$ of Davies ENDOR. As can be deducted from the Figure 3.9, the cause of such overestimation most likely lies in the largely overestimated contribution of oxygen’s $p_y$ orbital to the spin density.

Iron-oxygen distance in MbOH

With the $^1H$ hyperfine tensors obtained via three-pulse ESEEM, HYSCORE, and Davies ENDOR, we can calculate the distance between the iron and the hydroxyl proton by utilizing the point dipole approximation. As the configuration of the hydrogen with respect to the Fe-O bond distance is rather known, using these obtained distances we can also approximate the distance between the iron and the oxygen. This way we can compare our magnetic resonance results to Fe-O distances obtained by EXAFS and rRaman. Such data obtained by Timothy Yosca, along with DFT and our magnetic resonance measurements are presented in Table 3.1. Both DFT calculations and the magnetic resonance approximations fit the EXAFS data within 2% error. This result is very promising for all three EPR techniques deeming them a valid approach for distance determination of the Iron-hydroxyl proton distance. In addition, this result also indicates that the point-dipole approximation is most likely valid for this system.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Fe-O distance [Å]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Davies ENDOR</td>
<td>1.81 ± 0.04</td>
</tr>
<tr>
<td>3p ESEEM</td>
<td>1.84 ± 0.1</td>
</tr>
<tr>
<td>HYSCORE</td>
<td>1.83 ± 0.07</td>
</tr>
<tr>
<td>EXAFS</td>
<td>1.86 ± 0.1</td>
</tr>
<tr>
<td>rRaman</td>
<td>1.82 ± 0.06</td>
</tr>
<tr>
<td>DFT</td>
<td>1.81 ± 0.1</td>
</tr>
</tbody>
</table>

Table 3.1. Comparison of Fe-O distances obtained by magnetic resonance techniques, DFT, and rRaman

Summary and Conclusion

We have determined the hyperfine tensor of the hydroxyl $^1H$ nucleus of MbOH via different magnetic resonance techniques. All the techniques are in good agreement with each other. The obtained coupling constants can be indeed used to extract the Fe-H distances with great accuracy that can be correlated well with the Fe-O distance of MbOH. From all the used methods, Davies ENDOR gives
the best results with the HF coupling constants: $^1H A_{dip} = [5.5 \text{ 6.5} \text{ -12}]$ and $A_{iso} = 5 \text{ MHz}$. Therefore, these techniques can be deemed valid for distance determination of similar heme systems.

3.3 Materials and Methods:

*Myoglobin Sample Preparation*

Alkaline myoglobin was prepared by dissolving horse heart myoglobin from Sigma Aldrich (St. Louis, MO) in 50 mM borate buffer with 10% glycerol. The sample was frozen and stored at 77K, liquid nitrogen temperature.

*EPR Spectroscopy*

All ENDOR and ESEEM measurements were performed on the Elexsys E-560 CW/pulse X-band spectrometer equipped with a liquid helium cryostat from Oxford Instruments. Typical experimental conditions were as follows: temperature 10K, microwave frequency ~9.75 GHz, pulse MW power 1 kW. Davies ENDOR measurements utilized a 150W radiofrequency amplifier from Amplifier Research Company. Typical ENDOR pulse sequence: $p = 32\text{ns}$, $p_{\pi/2} = 16\text{ns}$ RF $\pi$ pulse, 14$\mu$s. To maximize bandwidth, all ESEEM measurements were performed using $p = 16\text{ns}$, $p_{\pi/2} = 8\text{ns}$ pulses. ESEEM data was processed in the following way: 1) baseline subtraction, 2) hamming window function, 3) zero-filling 4) fast-Fourier transform, 5) magnitude spectrum. All EPR simulations were performed using Easyspin. The Davies ENDOR spectra were fit via the ‘salt’ fitting function while all ESEEM data was fit via the ‘saffron’ function. The error was estimated to correspond to half the linewidth of the fit.

*Density Functional Theory*

All DFT calculations were performed using the Gaussian 03 computational package. Geometries were optimized via the B3LYP functional with the Pople basis set 6-311. The total hyperfine tensors were computed as a sum of the isotropic and dipolar contributions $A = A_{iso} + T$. The spin density isosurface was generated via the Gaussian 03 standalone application CUBEGEN and visualized using the Chemcraft program.
References

Appendix A

In Appendix A we present the derivation of the energy levels of an electron spin $S = \frac{1}{2}$ coupled to a nuclear spin $I = \frac{1}{2}$, where both the g-tensor and the hyperfine interaction are purely isotropic in nature. First, it is crucial to consider the basis set and all the relevant effective spin Hamiltonian operators that portray the system.

Such a system has a total of four states, in bra-ket notation ($|M_s, M_I>$) they are:

1) $M_s = \frac{1}{2}, M_I = \frac{1}{2}$ $\Rightarrow$ $|\frac{1}{2}, \frac{1}{2}>$
2) $M_s = \frac{1}{2}, M_I = -\frac{1}{2}$ $\Rightarrow$ $|\frac{1}{2}, -\frac{1}{2}>$
3) $M_s = -\frac{1}{2}, M_I = \frac{1}{2}$ $\Rightarrow$ $|-\frac{1}{2}, \frac{1}{2}>$
4) $M_s = -\frac{1}{2}, M_I = -\frac{1}{2}$ $\Rightarrow$ $|-\frac{1}{2}, -\frac{1}{2}>$


Computationally, the most convenient way to represent the effective spin Hamiltonian is in a matrix format. The angular momentum operators $J_x$, $J_y$, and $J_z$ in matrix form can be written as:

\begin{align*}
J_x & = \frac{1}{2} \left( J^+ + J^- \right) \\
J_y & = \frac{1}{2} i \left( J^+ - J^- \right) \\
J_z & = m |m> \\
J^+ |m> & = \frac{1}{\sqrt{2}} (J^+ - m - J^-) |m + 1> \\
J^- |m> & = \frac{1}{\sqrt{2}} (J^- - m + J^+) |m - 1> \\
J |m> & = m |m> \\
J^2 |m> & = J (J + 1 ) |m>
\end{align*}

Also,

\begin{equation}
J^2 |m> = J (J + 1 ) |m>
\end{equation}

For example, the $J_x$, $J_y$, $J_z$ for a spin of $\frac{1}{2}$ are just Pauli matrices $\sigma_{x,y,z}$ normalized to $1/2$:

\[
\begin{bmatrix} 1 & 0 \\ 0 & -1 \end{bmatrix} = \frac{1}{2} (1, 0 ; 0, -1) ; \quad \begin{bmatrix} 0 & 1 \\ 1 & 0 \end{bmatrix}; \quad \frac{1}{2} \begin{bmatrix} 0 & -i \\ i & 0 \end{bmatrix}
\]

\[
\begin{bmatrix} 1 & 0 \\ 0 & -1 \end{bmatrix} = \frac{1}{2} \frac{1}{2} \begin{bmatrix} 1 & 0 \\ 0 & -1 \end{bmatrix} \begin{bmatrix} 0 & 1 \\ 1 & 0 \end{bmatrix} = \frac{1}{2} \left( \begin{bmatrix} 0 & 1 \\ 1 & 0 \end{bmatrix} \times \begin{bmatrix} 0 & 1 \\ 1 & 0 \end{bmatrix} \right)
\]

In the case of $S= \frac{1}{2}$ coupled to a nuclear spin $I= \frac{1}{2}$, we first must define the coupled basis and use the matrix cross product to generate the corresponding operator matrix. The four basis functions where the first place indicates the electron spin and second the nuclear spin $|S,I>$ are:

\[
|\frac{1}{2}, \frac{1}{2}> , |\frac{1}{2}, -\frac{1}{2}> , |\frac{1}{2}, \frac{1}{2}> , |\frac{1}{2}, -\frac{1}{2}>
\]

We generate the $S_x, S_y, S_z, I_x, I_y,$ and $I_z$:

$S'_x$ (in coupled basis) = $S_x \times 1$ and $I'_x = 1 \times I'_x$ where $1$ is the unitary matrix.

\[
S'_x = \frac{1}{2} \begin{bmatrix} 0 & 1 \\ 1 & 0 \end{bmatrix} \times \begin{bmatrix} 0 & 1 \\ 1 & 0 \end{bmatrix} = \frac{1}{2} \begin{bmatrix} 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \\ 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \end{bmatrix}; \quad I'_x = \frac{1}{2} \begin{bmatrix} 1 & 0 \\ 0 & -1 \end{bmatrix} \times \begin{bmatrix} 1 & 0 \\ 0 & -1 \end{bmatrix} = \frac{1}{2} \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & 1 & 0 \end{bmatrix}
\]

Analogously:
The effective spin Hamiltonian for this simple system is comprised of the electron Zeeman, nuclear Zeeman and Hyperfine interaction.

\[ H_{\text{eff}} = H_{\text{EZ}} + H_{\text{NZ}} + H_{\text{HF}} \]

From equations 1.2.7, 1.2.8 and 1.2.9, we have:

\[ H_{\text{S}} = \mu_B \left( B_x B_y B_z \right) \begin{pmatrix} g_1 & 0 & 0 \\ 0 & g_2 & 0 \\ 0 & 0 & g_3 \end{pmatrix} \begin{pmatrix} S_x \\ S_y \\ S_z \end{pmatrix} - \mu_{\text{nag}} B_0 \begin{pmatrix} B_x B_y B_z \end{pmatrix} \begin{pmatrix} I_x \\ I_y \\ I_z \end{pmatrix} + \begin{pmatrix} S_x \\ S_y \\ S_z \end{pmatrix} \begin{pmatrix} A_x & 0 & 0 \\ 0 & A_y & 0 \\ 0 & 0 & A_z \end{pmatrix} \begin{pmatrix} S_x \\ S_y \\ S_z \end{pmatrix} \]

For instance the electron \( g \)-tensor is isotropic with \( g_x = g_y = g_z = 2 \) and the external magnetic field \( B \) is along the Z axis of the system with the magnitude \( B_0 \). Therefore the the Hamiltonian simplifies to:

\[ H_{\text{EZ}} = 2\mu_B B_0 S_z; \quad H_{\text{NZ}} = \mu_{\text{nag}} B_0 I_z; \]

\[ H_{\text{HF}} = E_x S_x - N_x I_x + H_{\text{HF}} \]

The \( H_{\text{HF}} \) is a sum of the x, y, and z components.

\[ H_{\text{HF}} = A_x S_x I_x + A_y S_y I_y + A_z S_z I_z. \]

\[ H_{\text{HF}} = \frac{A}{4} \begin{pmatrix} 0 & 0 & 1 \\ 0 & 1 & 0 \\ 1 & 0 & 0 \end{pmatrix} + \frac{A}{4} \begin{pmatrix} 0 & 0 & 1 \\ 0 & 1 & 0 \\ 1 & 0 & 0 \end{pmatrix} + \frac{A}{4} \begin{pmatrix} 0 & 0 & 1 \\ 0 & 1 & 0 \\ 1 & 0 & 0 \end{pmatrix} = \frac{A}{2} \begin{pmatrix} \frac{1}{2} & 0 & 0 \\ 0 & -\frac{1}{2} & 1 \\ 0 & 1 & -\frac{1}{2} \end{pmatrix} \]

therefore:

\[ H_{\text{eff}} = \frac{1}{2} \begin{pmatrix} E_x & 0 & 0 \\ 0 & E_x & 0 \\ 0 & 0 & -E_x \end{pmatrix} - \frac{1}{2} \begin{pmatrix} N_x & 0 & 0 \\ 0 & -N_x & 0 \\ 0 & 0 & N_x \end{pmatrix} + \frac{1}{4} \begin{pmatrix} 0 & -A & A \\ -A & 0 & -A \\ A & A & 0 \end{pmatrix} \]
To obtain the four true eigenvalues, the diagonalization of the non-diagonal matrix $M$ is necessary. To diagonalize it, we must obtain a diagonalization matrix $P$ where

$$P^{-1}MP = \begin{pmatrix} 
\lambda_1 & \cdots & 0 \\
\vdots & \ddots & \vdots \\
0 & \cdots & \lambda_2 
\end{pmatrix}. \quad (A.1.10)$$

After obtaining the appropriate eigenvalues, eigenvectors and the new basis the resulting eigenvalues are:

$$\lambda_1 = \frac{1}{2} E_x - \frac{1}{2} N_z - \frac{1}{2} A;$$

$$\lambda_2 = -\frac{1}{2} E_x + \frac{1}{2} N_z - \frac{1}{2} A;$$

$$\lambda_3 = \frac{1}{2} A - \left(\frac{1}{4} A^2 + (E_x + N_z)^2\right)^{\frac{1}{2}};$$

$$\lambda_4 = \frac{1}{2} A + \left(\frac{1}{4} A^2 + (E_x + N_z)^2\right)^{\frac{1}{2}}; \quad (A.1.11)$$

However, it is common practice to use the high field approximation where it is assumed that the electron Zeeman interaction is a few orders of magnitude larger in energy than the other terms ($E_z \gg N_z, A$). In this case the perturbation to the diagonal elements in equation A.1.9 is considered insignificant and the approximate effective eigenvalues from the lowest to highest energy are:

$$\lambda_1 = -\frac{1}{2} E_x - \frac{1}{2} N_z + \frac{1}{4} A; \quad \lambda_2 = -\frac{1}{2} E_x + \frac{1}{2} N_z - \frac{1}{4} A;$$

$$\lambda_3 = \frac{1}{2} E_x - \frac{1}{2} N_z - \frac{1}{4} A; \quad \lambda_4 = \frac{1}{2} E_x + \frac{1}{2} N_z + \frac{1}{4} A; \quad (A.1.12)$$
Appendix B

In Appendix B we present all the EasySpin simulations of the pulsed EPR data.

% HYSCORE of the strongly coupled 1H nucleus of MbOH

clear, clf

Sys.g = [1.83 2.15 2.60];
Sys.Nucs = "1H";
Sys.A = [5.3 5.9 -11.2] + 4.3;
Sys.Apa = [0 20 190]/180*pi;
%Exp.Sequence = 'HYSCORE';
Exp.Field = 315;
Exp.tau = 0.014;
Exp.dt = 0.016;
Exp.mwFreq = 9.5457;
Exp.ExciteWidth = 100;
Exp.Flip = [1 1 2 1];
Exp.Inc = [0 1 2 0];
Exp.t = [0.2 0 0 0.2]*1e-3;
Exp.nPoints = 300;
Opt.nKnots = 50;
saffron(Sys,Exp,Opt);
axis ([8 20 8 20]);

clear, clf

Sys.g = [1.83 2.15 2.60];
Sys.Nucs = '1H';
Sys.A = [5.3 5.9 -11.2] + 4.3;
Sys.Apa = [0 20 190]/180*pi;

%Exp.Sequence = 'HYSCORE';
Exp.Field = 375.4;
Exp.tau = 0.014;
Exp.dt = 0.016;
Exp.mwFreq = 9.5457;
Exp.ExciteWidth = 100;

Exp.Flip = [1 1 2 1];
Exp.Inc = [0 1 2 0];
Exp.t = [0.2 0 0 0.2]*1e-3;

Exp.nPoints = 300;

Opt.nKnots = 50;

saffron(Sys,Exp,Opt);
axis ([10 22 10 22]);

% ENDOR of the strongly coupled 1H nucleus of MbOH

clear, clf

% Spin system
Sys.S = 1/2;
Sys.g = [2.60 2.15 1.83];
Sys.Nucs = '1H';
Sys.HStrain = [100 100 100];
Sys.lwEndor = 0.5;
Opt.OriPreSelect = 1;
Sys.A = [5.5 6.5 -12] + S;
Sys.Apa = [0 55 155]*pi/180;

% Experimental Data
hold on
load MbOH2.txt
plot(MbOH2(:,1),MbOH2(:,2),’r-’);
plot(MbOH2(:,3),MbOH2(:,4),’r-’);
plot(MbOH2(:,5),MbOH2(:,6),’r-’);
plot(MbOH2(:,7),MbOH2(:,8),’r-’);
plot(MbOH2(:,9),MbOH2(:,10),’r-’);

% ENDOR experiment settings
Exp.mwFreq = 9.7416;
Exp.ExciteWidth = 1;
Opt.Enchancement = ’on’;
Fields = [273 300 322.6 341.7 367.8];
for iField = 1:numel(Fields) % loop over all field values
    Exp.Field = Fields(iField);
    Exp.Range = [0 (8+larmorfrq(Sys.Nucs,Exp.Field))];
    [freq(iField,:),spectra(iField,:)] = salt(Sys,Exp,Opt);
    FF(iField) = larmorfrq(Sys.Nucs,Exp.Field);
end
C = max(spectra,[],2);
for i = 1:numel(Fields)
    spectra(i,:) = spectra(i,:)/C(i);
end

% Plot theoretical ENDOR spectra
for j = 1:numel(Fields)
    plot(freq(j,:)-larmorfrq(Sys.Nucs,Fields(j)),spectra(j,:)+j-1);
end
% 3P ESEEM of the strongly coupled 1H nucleus of MbOH

clear, clf

Sys.g = [1.83 2.15 2.60];
Sys.Nucs = '1H';
Sys.A = [5.4 5.4 -10.8] +5.3;
Sys.Apa = [0 50 155]/180*pi;

Exp.Sequence = '3pESEEM';
Exp.Field = 289.8;
Exp.tau = 0.08;
Exp.dt = 0.016;
Exp.mwFreq = 9.5457;
Exp.ExciteWidth = 100;

Exp.t = [0.12 0.12 0.12]*1e-3;

%Exp.tp = [ 8 8]*1e-3;

Exp.nPoints = 256;

Opt.nKnots = 200;

saffron(Sys,Exp,Opt);
axis([8 20 0.4 .1]);

%=================================================================

clear, clf

Sys.g = [1.83 2.15 2.60];
Sys.Nucs = '1H';
Sys.A = [5.4 5.4 -10.8] +5.3;
Sys.Apa = [0 50 155]/180*pi;

Exp.Sequence = '3pESEEM';
Exp.Field = 311.2;
Exp.tau = 0.08;
Exp.dt = 0.016;
Exp.mwFreq = 9.5457;
Exp.ExciteWidth = 100;

Exp.t = [0.12 0.12 0.12]*1e-3;
%Exp.tp = [8 8 8]*1e-3;

Exp.nPoints = 256;

Opt.nKnots = 200;

saffron(Sys,Exp,Opt);
axis([8 20 0.4 .1]);