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Characterization of Oxygen Bridged Manganese Model Complexes using Multifrequency ^{17}O -Hyperfine EPR Spectroscopies and Density Functional Theory

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ABSTRACT: Multifrequency pulsed EPR data are reported for a series of oxygen bridged (μ -oxo/ μ -hydroxo) bimetallic manganese complexes where the oxygen is labeled with the magnetically active isotope ^{17}O ($I = 5/2$). Two synthetic complexes and two biological metallocofactors are examined: a planar bis- μ -oxo bridged complex and a bent, bis- μ -oxo- μ -carboxylato bridge complex; the di-manganese catalase, which catalyzes the dismutation of H_2O_2 to H_2O and O_2 ; and the recently identified manganese/iron cofactor of the R2lox protein, a homologue of the small subunit of the ribonucleotide reductase enzyme (class 1c). High field (W-band) hyperfine EPR spectroscopies are demonstrated to be ideal methods to characterize the ^{17}O magnetic interactions, allowing a magnetic fingerprint for the bridging oxygen ligand to be developed. It is shown that the μ -oxo bridge motif displays a small positive isotropic hyperfine coupling constant of about +5 to +7 MHz and an anisotropic/dipolar coupling of -9 MHz. In addition, protonation of the bridge is correlated with an increase of the hyperfine coupling constant. Broken symmetry Density Functional Theory is evaluated as a predictive tool for estimating hyperfine coupling of bridging species. Experimental and theoretical results provide a framework for the characterization of the oxygen bridge in Mn metallocofactor systems, including the water oxidizing cofactor of Photosystem II, allowing substrate/solvent interface to be examined throughout its catalytic cycle.

1 INTRODUCTION

The development of synthetic catalysts to address a diverse number of chemical problems, from water oxidation to energy storage processes, is turning to nature for inspiration. Biological systems use inexpensive, abundant materials to carry out complex, multi-electron chemistry. Of particular interest is the water oxidizing complex (WOC) of Photosystem II, which performs the four-electron, four-proton water-splitting reaction. Recent crystallographic data at atomic resolution has resolved the basic topology of the manganese tetramer that comprises the WOC.¹ Five oxygen atoms provide a network of bridges linking the four manganese atoms together.² This μ -oxo/hydroxo motif is a common structural feature of homo- and heteronuclear metal clusters, where such bridges govern the magnetic (exchange) coupling between the metal ions. Examples of manganese-containing bioinorganic cofactors include: a) the di-manganese catalase³⁻⁵, b) the recently identified Mn containing class Ib and 1c ribonucleotide reductases⁶⁻⁹, and c) the purple acid phosphatases.¹⁰ In each of these examples, the oxygen bridge represents either a substrate of the catalyzed reaction or a structural element introduced during catalyst activation.

Multifrequency electron paramagnetic resonance (EPR) spectroscopies, including multiresonance techniques such as Electron Spin Echo Envelope Modulation (ESEEM) and Electron-Nuclear Double Resonance (ENDOR), represent experimental methods to probe the electronic structure of the oxygen bridge via isotopic labeling with the paramagnetic ^{17}O nucleus. There are, however, a relatively small number of examples of oxo bridge cofactors characterized by these methods described in the literature. This is due to the low natural abundance (0.038%) of the ^{17}O isotope together

with its less than ideal magnetic properties, including i) a large nuclear spin $I = 5/2$, ii) a small nuclear g-factor ($g_n = -0.757$), and iii) a substantial nuclear quadrupole moment ($Q = -2.558 \text{ fm}^2$). These magnetic characteristics generally yield broad nuclear lines, which are often difficult to characterize via ESEEM. Simultaneously, such signals are expected to appear over the 0-20 MHz range (at low X/Q-microwave frequencies), rendering ENDOR both difficult to perform and to interpret, as the ^{17}O spectral lines overlap with other nuclei, e.g. $^1\text{H}/^2\text{H}$ and $^{14}\text{N}/^{15}\text{N}$. Nevertheless, X/Q-ENDOR has been successfully employed to characterize the μ -oxo bridge of two Fe containing metallocofactors: the mixed $\text{Fe}^{\text{II}}\text{Ni}^{\text{III}}$ ($S = 1/2$)¹¹ active site of a class of hydrogenase enzymes and the homometallic $\text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}$ ($S = 1/2$)¹² of the small subunit of class 1 ribonucleotide reductase. As expected, the reported ENDOR spectra are very broad, with hyperfine couplings in the range of 10-15 MHz which display large quadrupole splittings (3-11 MHz), a consequence of the large electric field gradient experienced by the coordinating ligands. Constrained fitting of these data sets was only achieved by resorting to two dimensional (2D)-techniques, where the magnetic field (orientation) dependence of the ENDOR signals was determined.¹²

Mixed valence complexes such as $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ and $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ form an important set of models used to benchmark spectroscopic studies of Mn metallocofactors. These complexes typically exhibit antiferromagnetic coupling leading to the ground state configuration of total electron spin (S_T) of $1/2$.^{4,13-16} The $S_T = 1/2$ state manifests itself in CW-EPR as a characteristic multiline signal centered at $g \sim 2$. This is in contrast to the Ni-Fe and Fe-Fe cofactor systems discussed above,^{11,12} both of which display a large g-anisotropy which

Figure 1. Structures of compounds studied in this work. A) Bent bis- μ -oxo, mono- μ -carboxylato $\text{Mn}^{\text{III}}(\mu\text{O})_2\text{-Mn}^{\text{IV}}$ DTNE complex;^{16,17} B) planar bis- μ -oxo $\text{Mn}^{\text{III}}(\mu\text{O})_2\text{-Mn}^{\text{IV}}$ BIPY complex^{18,20}; C) mono- μ -hydroxo, bis- μ -carboxylato $\text{Mn}^{\text{II}}(\mu\text{OH})\text{-Mn}^{\text{III}}$ PivOH complex;²¹ D, E) the Mn/Mn cofactor of *Thermus thermophilus* catalase (**MnCat**) poised in the $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ (bis- μ -hydroxo, mono- μ -carboxylato) and $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ (bis- μ -oxo, mono- μ -carboxylato) oxidation level;^{3,4} F) the $\text{Mn}^{\text{III}}\text{Fe}^{\text{III}}$ cofactor of the **R2lox** protein (mono- μ -hydroxo, bis- μ -carboxylato).^{9,22} The bridging μ -oxo oxygen atoms of the BIPY complex are identical whereas the bridging μ -oxo oxygen atoms of the DTNE and **MnCat** complexes are not strictly identical.

defines the width of the signal. The EPR spectrum of Mn complexes is instead defined by the ^{55}Mn hyperfine structure, as the intrinsic g-anisotropy is small. These properties however make the characterization of ligand hyperfine coupling of these complexes much more difficult, as 2D-experiments are harder to implement.

The Brudvig and Scholes laboratories were the first to characterize the μ -oxo bridge for a Mn complex using magnetic resonance techniques, specifically Q-band ^{17}O -ENDOR.²³ Upon ^{17}O labeling of the planar BIPY complex, they observed a broad structureless peak from which an isotropic coupling estimate of $A_{\text{iso}} = 12.8$ MHz was made, consistent with commensurate line-broadening observed in the EPR spectrum. No estimates were reported for either the hyperfine anisotropy or quadrupole coupling. A similar signal was later observed for the Mn catalase system, chemically poised in the ‘superoxidized’ $\text{Mn}^{\text{III}}/\text{Mn}^{\text{IV}}$ oxidation state.²⁴

Recently, W-band (95 GHz) EPR has been shown to be ideal for the study of nuclei with low nuclear g-values i.e. ^{14}N , ^{17}O .²⁵⁻²⁹ At these high frequencies, the nuclear Zeeman term is significantly larger than both the hyperfine and quadrupole terms of the spin Hamiltonian, and thus these two terms can be treated only to 1st order. An example is the solvation sphere of Ga^{III} complexes using Mims ENDOR.²⁷ Similarly, ESEEM techniques such as HYPERfine Sublevel CORrelation (HYSCORE) can be applied at high field to detect the ^{17}O nucleus,²⁶ a feat only previously achieved for selected Mo complexes.³⁰ In our own work we have used yet another hyperfine spectroscopy, ELection-electron DOuble Resonance (ELDOR)-detected NMR (EDNMR), a polarization transfer technique analogous to ENDOR.^{31,32} Using this technique at W-band, we could readily detect ^{17}O signals for Mn model complexes and metallocofactors,^{28,29,33} resolving the entire signal envelope. The values we measure for bridge coupling are very different, approximately 2-fold smaller than that of earlier studies. This has prompt-

ed us to perform an expanded multifrequency study for a series of oxygen bridged Mn complexes. We thus confirm that our earlier high field estimates are sound. In addition we can now show that the bridging ^{17}O hyperfine coupling is sensitive to the protonation state of the bridge. Various broken-symmetry density functional theory (BS-DFT) based methods for estimating hyperfine coupling of bridging ligands are evaluated with respect to their ability to reproduce the experimental data.

2. EXPERIMENTAL SECTION

2.1 DTNE and BIPY complexes: The mixed valence $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ complexes BIPY and DTNE were prepared using the procedure described in the literature.¹⁶⁻²⁰ Labeling of the μ -oxo bridge for each complex is described in **supp. info. S1**. The labeling efficiency was assessed using electrospray ionization (ESI) mass spectrometry: BIPY (56% double labeling, 37% mono labeled, 7% unlabelled), DTNE (57% double labeling, 36% mono labeled, 7% unlabelled), see **supp. info Figs S1.1-6**. EPR samples were prepared in either butyronitrile (DTNE) or 1:3 acetonitrile : dichloromethane (BIPY) as a solvent. The final concentration was about 500 μM . Sample tubes were degassed, sealed under vacuum and stored in liquid nitrogen.

2.2 Manganese catalase. Mn catalase (**MnCat**) protein from the thermophilic bacterium *Thermus thermophilus* was used for all EPR measurements. The protein was isolated and purified as described by Barynin et al.³⁴ The protein was stored in the form of a fine-crystalline precipitate in 50%-saturated ammonium sulphate at 4°C.^{34,35} To prepare the superoxidized state, the sample was first washed with aqueous buffer (10 mM K_2HPO_4 , pH 6.8). Two 50 μL fractions of the washed sample were then used to prepare the super-oxidized state in unlabeled and ^{17}O labeled water. Both were re-

suspended in 450 μL of KIO_4 buffer (5 mM) during one hour and washed with K_2HPO_4 buffer and concentrated to a volume of 20 μL . One sample was washed with 40 μL of H_2^{17}O buffer (x5, 90% ^{17}O enrichment), the other sample was washed with unlabelled water (x5) as control. ^{17}O labeled samples were either frozen to -80°C immediately after preparation or kept for 24 hours at room temperature.

2.3 The Mn/Fe cofactor of R2lox R2lox protein from the thermophilic bacterium *Geobacillus kaustophilus*, (*GkR2loxI*) was cloned and expressed as previously described by Griesel et al.⁹ The oxidized Mn/Fe cofactor was reconstituted as follows. 200 μM of apoprotein was incubated with 2 equivalents of MnCl_2 and 1 or 2 equivalents of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ in 100 mM HEPES-Na pH 7.0, 50 mM NaCl for 1 h at room temperature. Excess metal ions were removed by passing the sample through a HiTrap Desalting column equilibrated in 25 mM HEPES-Na pH 7.0, 50 mM NaCl. The $\text{Mn}^{\text{III}}\text{Fe}^{\text{III}}$ reconstituted protein was concentrated to 0.4 – 0.6 mM and exchanged into H_2^{17}O -based buffer (25 mM HEPES-Na pH 7.0, 50 mM NaCl in 90% H_2^{17}O) by four rounds of dilution and concentration. 50% (v/v) glycerol was added to the samples before transfer into sample holders and flash cooling in liquid nitrogen, so that the final protein concentration was 0.2 – 0.3 mM.

2.4 X-, Q-band EPR experiments. X-Band CW and pulse EPR measurements were performed using a Bruker ELEXSYS 580 spectrometer, equipped with an Oxford Instruments ESR 935 cryostat and ITC4 temperature controller. Q-band pulse EPR measurements were performed at 40 K using a Bruker ELEXSYS E580 Q-band pulse EPR spectrometer, equipped with homebuilt TE_{011} microwave cavity,³⁶ Oxford-CF935 liquid helium cryostat and ITC-503 temperature controller. All ENDOR data were collected using an external home-built computer console (Spec-Man4EPR control software³⁷) with the ELEXSYS E580 X-band pulse EPR spectrometer, coupled to an external RF generator and RF amplifier (ENI 5100L). Electron spin echo-detected (ESE) field-swept spectra were measured using the pulse sequence: $t_p - \tau - 2t_p - \tau - \text{echo}$. The length of $\pi/2$ microwave pulse was generally set to $t_p = 8$ ns (X-band), 36 ns (Q-band). The interpulse distance was varied in the range $\tau = 200$ –500 ns (X/Q-band). Using either an FID detection or Hahn echo sequence did not significantly change the measured pulse spectrum. The relatively slow T_1 relaxation time observed for these complexes required the measurement temperature to be above 30 K for efficient data collection (shot repetition rates of 1 ms).

Davies type ^{17}O ENDOR spectra were acquired using the pulse sequence: $t_{\text{inv}} - t_{\text{RF}} - T - t_p - \tau - 2t_p - \tau - \text{echo}$ using the length of inversion microwave π pulse of $t_{\text{inv}} = 128$ ns, radio frequency π pulse length of $t_{\text{RF}} = 6$ μs . The length of $\pi/2$ microwave pulse in the detection sequence was generally set to $t_p = 64$ ns and the interpulse delays to $T = 1.5$ μs and $\tau = 468$ ns. Mims type ^{17}O ENDOR spectra were collected using the pulse sequence: $t_p - \tau - t_p - t_{\text{RF}} - T - t_p - \tau - \text{echo}$, with $t_p = 8$ ns, $t_{\text{RF}} = 6$ μs , $\tau = 300$ –500 ns and $T = 2$ μs . The RF frequency was swept 20 MHz around the ^{17}O -Larmor frequency of about 3 MHz (340 mT) in 50 kHz steps. All ENDOR measurements were performed using the random (stochastic) acquisition technique as described in Epel et al.³⁷

2.5 W-band EPR experiments. High-field EPR experiments were performed using a W-band EPR spectrometer (Bruker Elexsys E680), equipped with a homebuilt ENDOR microwave cavity

optimized for maximum RF performance at low RF frequencies ($< 100\text{MHz}$, optimum performance at 20 MHz).²⁹ Electron spin echo-detected (ESE) field-swept spectra were measured using the pulse sequence: $t_p - \tau - 2t_p - \tau - \text{echo}$ with $t_p = 24$ ns and $\tau = 200$ –500 ns. HYSCORE spectra were collected using the $t_p - \tau - t_p - t_1 - 2t_p - t_2 - t_p - \tau - \text{echo}$ sequence with $t_p = 20$ ns, $\tau = 396$, 408, 420 ns and $t_1, t_2 = 100$ –1124 ns in 8 ns steps. Davies type ^{17}O -ENDOR spectra were collected using the $t_{\text{inv}} - t_{\text{RF}} - T - t_p - \tau - 2t_p - \tau - \text{echo}$ sequence with $t_{\text{inv}} = 128$ ns, $t_p = 64$ ns, $t_{\text{RF}} = 15$ μs , $T = 4$ μs and $\tau = 600$ ns. T-averaged Mims type ^{17}O ENDOR spectra were collected using the $t_p - \tau - t_p - t_{\text{RF}} - T - t_p - \tau - \text{echo}$ sequence with $t_p = 24$ ns, $t_{\text{RF}} = 15$ μs , $T = 4$ μs , and $\tau = 164$ –332 ns in 12 ns steps. The RF frequency was swept 23.5 MHz around the ^{17}O -Larmor frequency of about 19.7 MHz (3.4 T) in 200 kHz steps. EDNMR measurements were performed using the pulse sequence: $t_{\text{HTA}} - T - t_p - \tau - 2t_p - \tau - \text{echo}$. The high tuning angle (HTA) microwave pulse was applied at microwave frequency ν_{mw} . The detection Hahn echo pulse sequence $t_p - \tau - 2t_p - \tau - \text{echo}$ at microwave frequency $\nu_{\text{mw}}^{(0)}$ matched to the cavity resonance was set 2 μs post HTA pulse to ensure the decay of the electron spin coherence. The pulse length for a detection $\pi/2$ pulse $t_p = 40$ –200 ns and the pulse separation $\tau = 600$ ns were generally set. The echo was integrated ≈ 1000 ns around its maximum. The spectra were acquired continuously sweeping the HTA frequency ν_{mw} at fixed B_0 . A low-power HTA microwave pulse of $t_{\text{HTA}} \approx 8$ μs length was used to minimize the width of the central hole. Tabulated pulse EPR parameters for each experiment are given in **supp. info. Table S2.1**

2.6 Spectral simulations. EPR/ ENDOR/ HYSCORE/ EDNMR spectra were simultaneously fit assuming an effective spin $S = 1/2$ ground state (see Theory section 3.2). The electron Zeeman, nuclear Zeeman and hyperfine terms were treated exactly. Spectral simulations were performed numerically using the EasySpin package^{38,39} in MATLAB.

2.7. Geometry optimizations and EPR parameter calculations using BS-DFT. The models were optimized in their high spin states using the B3LYP functional^{40–42} along with the D3 dispersion corrections from Grimme with the Becke–Johnson damping scheme.^{43,44} The zero-order regular approximation (ZORA) was used to account for relativistic effects while the conductor-like screening model (COSMO) was used to model solvent effects.^{45–49} For small molecule complexes acetonitrile was used as the solvent ($\epsilon = 36.60$). The ZORA-recontracted versions of the def2-TZVP(-f) basis sets were used for all atoms except C and H, for which the corresponding ZORA SVP basis sets were employed.^{50,51} Optimizations took advantage of the resolution of the identity approximation for Coulomb exchange (RI-J)⁵² and the chain-of-spheres approximation (COSX) for Hartree–Fock exchange,⁵³ as implemented in ORCA,⁵⁴ with the decontracted auxiliary def2-TZVPP/J Coulomb fitting basis sets.⁵⁵ Tight SCF convergence criteria were used throughout. All broken-symmetry single-point calculations and all calculations of reported EPR parameters were computed with the TPSSH functional⁵⁶ and ZORA def2-TZVP(-f) basis sets on all atoms. Hyperfine tensors for the N, O, Mn, and Fe atoms were calculated from the broken-symmetry solution using the same methodology as described previously for a model $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ PivOH and R2lox $S = 1/2$ complexes,^{22,57} with locally dense integration grids and basis sets extended in the core region.

2.8 Construction of protein cluster models. For protein bioinorganic complexes cluster models were constructed from available published crystal structures (2V8U and 4HR0 for dimanganese catalase⁵ and R2lox,⁹ respectively). Cluster models were constructed using all transition metal (Mn or Fe) coordinating residues, μ -oxo/hydroxo bridges, and water molecules from chain A of the crystal structures. Amino acids were truncated at the α -C while the fatty acid ligand of R2lox was truncated as a three carbon atom hydrocarbon chain (see **Fig. 1**). Hydrogen atoms were then added to fill in the remaining valence, after which a preliminary optimization of only the hydrogen atoms was performed. Subsequent to the hydrogen-only-optimization the positions of the hydrogen atoms on the terminal carbon atoms were constrained and a full geometry optimization with all other atoms free was performed. These models were then used in the single point broken-symmetry and EPR parameter calculations. All calculations of the protein models used a dielectric constant of $\epsilon = 4.0$ in conjunction with COSMO. In all other aspects the optimizations of the cluster models followed the same methodology as for the small molecule complexes.

3 THEORY

3.1 The spin Hamiltonian formalism. Here we consider an antiferromagnetically exchange coupled $\text{Mn}^{\text{III}}\text{-O}_2\text{-Mn}^{\text{IV}}$ dimer. A basis set that describes the Mn-dimer spin manifold can be built from the product of the eigenstates of the interacting spins:

$$|S_1 M_1\rangle \otimes |S_2 M_2\rangle \otimes |I_1 m_1\rangle \otimes |I_2 m_2\rangle \quad (\text{Eq. 1})$$

Here S_i refers to the electronic spin state of Mn, M_i refers to the electronic magnetic sub-level, I_i refers to the nuclear spin state of ^{17}O , m_i refers to the nuclear magnetic sub-level of ^{17}O . The metal (^{55}Mn) spin quantum numbers are excluded for simplicity. The full basis set is given in the **supp. info. S6**. S_i takes the value 2 for Mn^{III} and 3/2 for Mn^{IV} ; M_i takes the values: $S_i, S_i-1, \dots, 1-S_i, -S_i$; I_i takes the value 5/2 for ^{17}O and m_i takes the values $-I_i, 1-I_i, \dots, I_i-1, I_i$. The Spin Hamiltonian that describes the spin manifold of the ^{17}O labeled Mn dimer is:

$$\begin{aligned} \hat{H} = & -2J\vec{S}_1 \cdot \vec{S}_2 + \sum_{i=1,2} \vec{S}_i \cdot \mathbf{d}_i \cdot \vec{S}_i \\ & + \beta \sum_{i=1,2} \vec{B}_0 \cdot \mathbf{g}_i \cdot \vec{S}_i - g_n \beta_n \sum_{k=1,2} \vec{B}_0 \cdot \vec{I}_k \\ & + \sum_{i=1,2} \sum_{k=1,2} \vec{S}_i \cdot \mathbf{a}_{i,k} \cdot \vec{I}_k + \sum_{k=1,2} \vec{I}_k \cdot \mathbf{p}_k \cdot \vec{I}_k \end{aligned} \quad (\text{Eq. 2})$$

It contains: i) an electron-electron spin coupling term for the Mn-Mn interaction; ii) a fine structure term for each Mn ion; iii) an electronic Zeeman term for each Mn ion; iv) an nuclear Zeeman term for each ^{17}O nucleus; v) an electron-nuclear hyperfine term for each ^{17}O nucleus; and vi) a nuclear quadrupole term for each ^{17}O nucleus.

3.2 The effective spin $1/2$ Ground State. The electronic coupling between the two Mn ions in mixed valence Mn dimers is usually dominated by the through bond exchange interaction and is sufficiently large that the spin manifold can be treated within the strong exchange limit. In this instance the exchange interaction between the two Mn ions is significantly larger than any other term of the spin Hamiltonian.^{14,19,58} The resultant electronic spin states of the manifold are then adequately described by a single quantum number, the total spin (S_T).⁵⁹ The ‘multiline’ EPR signal observed is

derived from only one total spin state, the ground state of the spin manifold with total spin $S_T = 1/2$. The basis set that describes this subspace takes the form:

$$|S_T M I_1 I_2 m_1 m_2\rangle \quad (\text{Eq. 3})$$

Where M takes all half-integer values: $-\frac{1}{2} \leq M \leq \frac{1}{2}$; m_i (where $i = 1, 2$) takes all half integer values: $-\frac{5}{2} \leq m_i \leq \frac{5}{2}$. The effective Spin Hamiltonian that describes the ground state of the spin manifold ($S_T = 1/2$) is:

$$\begin{aligned} \hat{H} = & +\beta \vec{B}_0 \cdot \mathbf{G} \cdot \vec{S}_T - g_n \beta_n \sum_{k=1,2} \vec{B}_0 \cdot \vec{I}_k \\ & + \sum_{k=1,2} \vec{S}_T \cdot \mathbf{A}_k \cdot \vec{I}_k + \sum_{k=1,2} \vec{I}_k \cdot \mathbf{p}_k \cdot \vec{I}_k \end{aligned} \quad (\text{Eq. 4})$$

It contains: i) the electronic Zeeman term for the total electronic spin; ii) nuclear Zeeman terms for each ^{17}O nucleus; iii) electron-nuclear hyperfine terms for each ^{17}O nucleus and; iv) nuclear quadrupole terms for each ^{17}O nucleus. Note that throughout the text lower case letters are used for spin Hamiltonian parameters in the uncoupled representation (site parameters) were as upper case letters are used for spin Hamiltonian parameters in the coupled representation (measured projected parameters).

3.3 Hyperfine tensor decomposition. The ligand terms described here are described in terms of two tensor components, the isotropic coupling A_{iso} and the anisotropic coupling A_{aniso} (ignoring the anti-symmetric term):

$$\mathbf{A} = A_{\text{iso}} \mathbf{I} + \mathbf{A}_{\text{aniso}} \quad (\text{Eq. 5})$$

Where A_{iso} is the trace of the hyperfine tensor and I is the identity matrix. A_{aniso} in its diagonalized form is defined here in terms of two parameters D and η :

$$\mathbf{A}_{\text{aniso}} = D \cdot \begin{bmatrix} -(1+\eta) & 0 & 0 \\ 0 & -(1-\eta) & 0 \\ 0 & 0 & 2 \end{bmatrix} \quad (\text{Eq. 6})$$

The rhombicity (η) is expected to be small for terminal ligands, but may be large for bridging ligands due to the interaction with more than one spin center/fragment (see **supp. info. S7**).

3.4 Isotropic spin projections. A mapping of the spin subspace in section 3.2 onto the original basis set as described in section 3.1 can be made. This allows the intrinsic g and hyperfine tensors ($\mathbf{g}$, $\mathbf{a}_{i,k}$, see eq. 2) to be calculated from the effective G and hyperfine tensors ($\mathbf{G}$, $\mathbf{A}_i$, see eq. 4).⁵⁸⁻⁶⁰ These weighting factors are termed spin-projection coefficients. The isotropic spin projection coefficients (ρ_i) are defined in terms of the ratio of the spin expectation value S_z of the i^{th} spin center and the total spin S_T :

$$\rho_i = \frac{\langle S_{i,z} \rangle}{S_T} \quad (\text{Eq. 7})$$

For a two spin system (S_1, S_2) there exists an explicit solution for the two spin projections (ρ_1, ρ_2) which define this mapping.⁵⁹

$$\rho_{i=1,2} = \frac{S_i(S_i+1) - S_{3-i}(S_{3-i}+1) + S_T(S_T+1)}{2S_T(S_T+1)} \quad (\text{Eq. 8})$$

Expressing then the effective isotropic G and hyperfine values (A_k) yields:⁵⁹

$$G = \sum_{i=1,2} \rho_i g_i \quad \& \quad A_{k=1,2} = \sum_{i=1,2} \rho_i a_{i,k} \quad (\text{Eqs. 9})$$

For example: for a $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ dimer, $S_1(\text{Mn}^{\text{III}}) = 2$; and $S_2(\text{Mn}^{\text{IV}}) = 3/2$ Eqs. 8 yields isotopic spin projection values of $\rho_1 = 2$ and $\rho_2 = -1$, respectively. Inserting these values in Eqs.9 yields formulae for the effective G and hyperfine tensors $G = 2g_1 - g_2$, $A_1 = 2a_{0,11} - a_{0,12}$; $A_2 = 2a_{0,12} - a_{0,22}$.^{60,61}

3.5 Broken symmetry-DFT calculation of hyperfine coupling constants. The projection of the hyperfine coupling constants calculated using broken symmetry DFT (BS-DFT) for terminal ligands coordinated to a metal spin center has been discussed previously in the literature.⁶² As described above, the measured (projected) hyperfine coupling for a particular nucleus (k) represents the site hyperfine coupling (a_k) multiplied by the spin projection factor ρ_i associated with the metal (i^{th} spin center) it is ligated to.

$$A_k = \rho_i a_k \quad (\text{Eq. 10})$$

with the spin projection (ρ_i) calculated as the ratio of spin expectation value S_z of the broken symmetry wave-function and the total spin quantum number (S_T). It is noted that the site hyperfine is not directly calculated using BS-DFT. The as-calculated “raw” BS-DFT value, labelled with the superscript ‘BS’ (a_k^{BS}), must first be re-scaled to account for the BS calculation being incorrectly scaled; in the broken symmetry solution the calculated hyperfine values reflect the coupling of the nuclear spin k to a fictitious $S = 1/2$ electronic spin. To calculate the site hyperfine this value must be scaled to taken into account the actual spin state of the metal which ligates the nuclear spin, i.e. the raw hyperfine is by the ratio of the expectation value of fictitious electronic spin operator (i.e. M_z), divided by the site spin quantum number (S_i).

$$a_k = \pm a_k^{\text{BS}} \frac{\langle S_z \rangle^{\text{BS}}}{S_i} \quad (\text{Eq. 11})$$

For the calculations presented here S_i refers the spin quantum number of the spin fragment the nuclear spin couples to, in this instance either the Mn^{III} ion ($S_{i=1} = 2$) or the Mn^{IV} ion ($S_{i=2} = 3/2$).

$\langle S_z \rangle^{\text{BS}}$ is the spin expectation value for the broken system solution. This takes only one value i.e. $1/2$, as only for the ground state configuration is considered. Note that the spin density (Fermi contact interaction) appears only once in equation 11; it is contained in the raw hyperfine value. The hyperfine values are signed quantities. Assuming a fixed sign for the site hyperfine coupling, the projected hyperfine of nucleus (k) will be either positive or negative depending on whether it is ligated to the spin center (forms part of the spin fragment) that carries either majority (α) or minority (β) spin. The formalism shown in Eq.11 generalizes for all components of the hyperfine tensor. For more information on the projection of calculated BS-DFT values for comparison with experiment we refer the reader to refs.⁶²⁻⁶⁵ For bridging ligands the k^{th} nucleus cannot be assigned to a single spin-centered system. This then poses a problem as to how to best interpret the raw hyperfine values. Although at present no rigorous method exists to achieve an appropriate spin projection as described above for terminal ligands, here we have evaluated three possible approximations.

i) As the spin density of the bridging ligand cannot be sensibly decomposed into two site interactions with each spin fragment, and the BS-DFT solution is correctly scaled in terms of the total spin multiplicity, the raw hyperfine value (a_k^{BS}) might be used as a first approximation to the correct value. Importantly, as a_k^{BS} scales with the Fermi contact term, this parameter should at least reproduce the trend in hyperfine couplings seen across the different models. Thus this serves as the simplest way to validate if BS-DFT is appropriate for the calculation of bridging hyperfine coupling constants.

ii) The second approach is simply to average the two projected hyperfine couplings for the bridging ligand, as has been proposed in earlier literature by Noodleman and coworkers.^{66,67} In this instance, the raw hyperfine value (a_k^{BS}) is projected for each metal ion (spin fragment) individually (Eqs. 10-11), summed and averaged

$$A_{k=1,2} = \frac{\rho_1 a_{1k} + \rho_2 a_{2k}}{2} \quad (\text{Eq. 12})$$

For this method as same raw hyperfine value is used to calculate the two site hyperfine values Eq. 12 can be re-expressed as:

$$A_{k=1,2} = \frac{a_k^{\text{BS}}}{2} \left(\frac{\langle S_z \rangle^{\text{BS}}}{S_1} |\rho_1| + \frac{\langle S_z \rangle^{\text{BS}}}{S_2} |\rho_2| \right) \quad (\text{Eq.13})$$

For a $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ complex equation 13 simply reduces to:

$$A_{k=1,2} = \frac{5}{12} a_k^{\text{BS}} \quad (\text{Eq. 14})$$

And the corresponding $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ complex and the isoelectronic $\text{Mn}^{\text{III}}\text{Fe}^{\text{III}}$ complex:

$$A_{k=1,2} = \frac{4}{10} a_k^{\text{BS}} \quad (\text{Eq. 15})$$

iii) The third approach directly follows from the spin projection mapping used to scale hyperfine parameters from the uncoupled to the coupled spin Hamiltonian representation. As with the second approach it does however assume that the calculated hyperfine value (a_k) represents the site hyperfine coupling for the bridging ligand interacting with each metal site. In practice this cannot be the case as these two interactions involving different orbitals. Thus *ab initio* methods, beyond the scope of this work, are necessary to decompose the calculated hyperfine into its two components for a completely robust methodology. As implied from Eq. 7, the projected hyperfine is the sum of the two site interactions:

$$A_{k=1,2} = \sum_{i=1,2} \rho_i a_{ik} = \rho_1 a_{1k} + \rho_2 a_{2k} \quad (\text{Eq. 16})$$

As per Eq.12, the raw hyperfine value is the same for both terms of Eq. 16, and as such it can be re-expressed as:

$$A_{k=1,2} = a_k^{\text{BS}} \left(\pm \frac{\langle S_z \rangle^{\text{BS}}}{S_1} \rho_1 \mp \frac{\langle S_z \rangle^{\text{BS}}}{S_2} \rho_2 \right) \quad (\text{Eq. 17})$$

For a $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ complex equation 17 simply reduces to:

$$A_{k=1,2} = \frac{5}{6} a_k^{\text{BS}} \quad (\text{Eq. 18})$$

And the corresponding $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ complex and the isoelectronic $\text{Mn}^{\text{III}}\text{Fe}^{\text{III}}$ complex:

$$A_{k=1,2} = \frac{4}{5} a_k^{BS} \quad (\text{Eq. 19})$$

Thus it can be readily inferred that method (i) will always give the largest hyperfine estimate, method (ii) will always give the smallest hyperfine estimate, which for these systems is approximately half the raw hyperfine coupling, and method (iii) will yield a value in between.

4. RESULTS AND DISCUSSION

4.1 EPR SPECTROSCOPY

4.1.1 Multifrequency CW/pulse EPR. CW and pulse EPR data of the $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ DTNE complex in frozen solution of butyronitrile are presented in **Figure 2A**. A 16 line EPR pattern of approximately the same total spectral breadth was observed at all microwave frequencies.^{15,16,68} Such spectra are typical for mixed valence $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ complexes in which the g-anisotropy is small and the two metal hyperfine couplings define the structure of the signal. The EPR line-width of the 16 major lines is narrow and additional

side lines are resolved throughout the EPR spectrum (FWHM ≈ 3 mT – X-band). This side structure is more readily observed at X-band, suggesting field inhomogeneity (g-strain) contributes to the EPR line-width at higher frequency. Similar results were obtained for the ^{16}O labeled BIPY complex, see **supp. info. S2**. The ^{17}O label was incorporated via isotope exchange with ^{17}O labeled water, see **supp. info. S1**. As expected, incorporation of the ^{17}O isotope caused a significant increase in the EPR line-width observed at all microwave frequencies. This is because each spectral line splits due to the new hyperfine interaction between the electron spin and the ^{17}O nucleus. This splitting is however smaller than the spectral line-width, and as such manifests only as an EPR line broadening. The extent of this broadening is most readily observed at X-band, (FWHM ≈ 4 mT – X-band). The same labeling procedure was repeated with ^{18}O labeled water to demonstrate that oxygen atom exchange does not influence the intrinsic EPR line-width. As expected the ^{18}O labeled DTNE complex was identical to the unlabeled ^{16}O DTNE complex, as both the ^{16}O and ^{18}O isotope do not have a nuclear spin. Fig. 2 shows a spin Hamiltonian simulation to account for the observed line-width broadening. These simulations assume a ^{17}O labeling efficiency of 57% double, 36% mono and 7% unlabeled, as determined by ESI mass spectrometry, see **supp info. S1**.

Figure 2. A) CW X-band EPR spectra of the DTNE complex containing the ^{16}O , ^{17}O and ^{18}O isotopes in frozen solution of butyronitrile (40 K). B, C) Corresponding Q-band and W-band pulse EPR data. Pulse EPR data are pseudomodulated with a Bessel function of the 1st kind. Spin Hamiltonian simulations are shown by the red dashed lines superimposing the data. All experimental parameters are given in the **supp. info S2**, simulation parameters in **supp. info. S6**. Asterisks (*) indicate background resonator signals.

The G tensor and ^{55}Mn hyperfine tensor values used for the simulation are approximately the same as those determined previously.^{15,16,68} The line broadening seen upon ^{17}O label incorporation was reproduced with an equivalent hyperfine coupling for the two μ -oxo bridges of ≈ 15 MHz. Approximately the same line broadening was observed for the ^{17}O -BIPY complex as compared to the ^{16}O -BIPY complex. This was modeled with an equivalent hyperfine coupling for the two μ -oxo bridges of ≈ 13 MHz, equivalent to the value reported by Usov et al.,²³ see **supp. info S2**.

4.1.2 X-band Mims/Davies ^{17}O -ENDOR. To further characterize the ^{17}O hyperfine interaction both Mims and Davies ENDOR experiments were performed for the ^{16}O and ^{17}O labeled DTNE complex (**Fig. 3**). For the ^{16}O sample two sharp lines at 5.5 MHz and 7.5 MHz were observed using Mims ENDOR, split by ≈ 2 MHz. The doublet had a pronounced magnetic field dependence, with the center of the doublet shifting 0.5–1.0 MHz to higher frequency when comparing to the ENDOR spectrum, collected at the high and low edge of the EPR signal. This doublet is consistent with its assignment to the ^{14}N ligand(s). These signals were not as readily observed in the corresponding Davies ENDOR experiment, presumably because they exhibit only a small hyperfine coupling and are thus likely suppressed.

For the corresponding ^{17}O labeled DTNE complex a new, broad signal was observed in both Mims and Davies ENDOR spectra, extending from less than 4 MHz to at least 10 MHz with peak maximum at ≈ 6 MHz. Unlike the background ^{14}N doublet, the frequency position/line-shape of this new signal does not significantly change with the external magnetic field, consistent with its assignment to an ^{17}O ligand in the strong hyperfine coupling limit ($|A| \gg 2\nu_n$). It is suspected that this turning point only represents half of the ^{17}O -ENDOR signal, specifically the plus (ν^+) branch of the total signal, see section 4.1.8. Assuming the hyperfine coupling is isotropic and quadrupole splitting is ignored, the position of the ^{17}O -ENDOR signal is given by the equation $\nu^+ = |A_{\text{iso}}|/2 + \nu_n$, where A_{iso} is the isotropic hyperfine coupling and ν_n the Larmor frequency of the ^{17}O nucleus at a given magnetic field [$\nu_n(^{17}\text{O}) = 2.02$ MHz, $B_0 = 350$ mT]. This yields a ^{17}O hyperfine coupling estimate of ≈ 7.5 MHz, significantly smaller than that inferred from the linewidth broadening seen in the EPR spectrum.

4.1.3 W-band ^{17}O -EDNMR. The EPR line-shape analysis and X-band ENDOR data presented above clearly do not give the same hyperfine coupling value. The reason for this however is already evident from the ENDOR data. These spectra resolve very broad ^{17}O spectral lines indicating that the hyperfine tensor has a large anisotropic contribution. They may also indicate that the nuclear quadrupole interaction is large. To better determine these parameters, EPR measurements at W-band frequency were performed. At high field (3.4 T), the nuclear Zeeman term for all 1st coordination sphere $^{17}\text{O}/^{14}\text{N}$ ligands is significantly larger as compared to the hyperfine estimate, falling now in the weak coupling regime ($|A| < 2\nu_n$). Spectral peaks should now appear as a simpler pattern about the Larmor frequency split by the hyperfine. It is also noted that the Larmor frequency of ^{17}O [$\nu_n(^{17}\text{O}) = 19.6$ MHz, $B_0 = 3.4$ T] is significantly different from that of ^{14}N [$\nu_n(^{14}\text{N}) = 10.5$ MHz], thus allowing both components to be readily distinguished from each other, as they appear in different frequency regions. ENDOR measurements at W-band were attempted for both the DTNE and BIPY complex, however spectra of sufficient quality for analysis could not be obtained.

Figure 3. A) X-band echo detected EPR spectrum of the DTNE complex. B) Corresponding ^{17}O -ENDOR data collected at three magnetic field positions marked in A. The colored traces represent Mims ENDOR spectra of the ^{17}O labeled (blue) and ^{16}O labeled (black) complex. The difference is shown in green. The black trace at the bottom represents corresponding Davies ENDOR spectra. For all data a 3pt point smoothing function was applied. I, II, III indicate the three magnetic field positions (B_0) where the ENDOR spectra were collected. All EPR parameters are given in the materials and methods section.

In contrast to ENDOR, high quality EDNMR spectra were obtained for both complexes. The 2D EDNMR surface of the ^{17}O labeled mixed-valent $\text{Mn}^{\text{III}}\text{-}\mu\text{O}_2\text{-Mn}^{\text{IV}}$ DTNE complex is shown in **Fig. 4**. Key advantages of this technique were outlined in our earlier study.²⁹ In particular, the high sensitivity of this technique allows 2D experiments, where the EDNMR data are collected across the EPR spectrum. Control ^{16}O DTNE complex data are shown in **black** in **Fig. 4B**, resolving signals attributable to a ^{14}N ligand. Both single and double quantum transitions are observed, centered at 10.4 and 20.8 MHz respectively. The single quantum transitions are split by the hyperfine coupling ($A \approx 10$ MHz), whereas the double quantum transitions split by twice the hyperfine coupling. The ^{14}N peaks are characteristically narrow with peak widths of FWHM 3 MHz, similar to that seen for the BIPY complex,²⁹ and represent the strongly coupled axial ligand which sits along its Jahn-

Figure 4. **A**) Two dimensional representation (contour map) of the W-band EDNMR spectrum of the ^{17}O labeled **DTNE** complex. **B**) A comparison of the EDNMR signal seen for the ^{17}O labeled complex (blue lines) compared to unlabeled complex (black lines) at three field positions **I**, **II**, **III** marked at the top of panel **A**. Spectral represent the average of the left and right hand side of the raw EDNMR spectrum.

Teller axis of the Mn^{III} ion.⁶⁹ The remaining equatorial ^{14}N ligands of the Mn^{III} and all ^{14}N ligands of the Mn^{IV} appear as a ‘matrix’ line centered at the ^{14}N Larmor frequency. The corresponding ^{17}O labeled **DTNE** complex EDNMR data are shown in blue in Fig. 4B. The ^{17}O signal observed is significantly different from that of the background ^{14}N signal. It is much broader and does not resolve any structure. The single quantum ^{17}O transitions does not appear as a doublet centered about the Larmor frequency of ^{17}O [$v_n(^{17}\text{O}) = 19.4$ MHz, $B_0 = 3.37$ T]. It instead is a broad envelope, where a two peak structure is only observed for the EDNMR spectrum measured on the low field edge (Fig. 4B, I). An apparent splitting is all but lost for the EDNMR spectrum, measured on the high field edge (Fig. 4B, III), and the spectrum in the center represents an average of the two edge spectra (Fig. 4B, II). While a discrete peak structure is absent for the ^{17}O signal, the total width of the envelope still provides a complete description of the ^{17}O hyperfine tensor. Spin Hamiltonian simulations are shown in the **supp. info. S6**. However, as is often the case, there are two equally valid interpretations of this dataset, which differ depending on the relative signs of the isotropic and anisotropic hyperfine terms. This is because the transitions strongly overlap such that only two hyperfine tensor components are observed in isolation. In the instance where A_{iso} and D take opposite sign, A_{iso} is small and the A_{aniso} tensor is approximately axial (Table 1). In contrast, if A_{iso} and D take the same sign, A_{iso} is instead large and A_{aniso} is highly rhombic. Furthermore, the two different tensor geometries assign the unique tensor axis differently. In the case of solution 1, the unique tensor axis is the smallest in absolute magnitude and coincides with g_z , whereas in solution 2, it is instead largest in absolute magnitude and coincides with g_x (Table 1). The corresponding 2D-EDNMR surface for the **BIPY** complex was previously published in Rapatskiy et al.²⁹

The EDNMR data for both complexes are very similar in terms of the structure of the ^{17}O signal profile and its dependence on the magnetic field. However, the **BIPY** complex is systematically broader by about ≈ 2 MHz. Spin Hamiltonian simulations of the **BIPY** EDNMR data are also shown in **supp. info. S6** and all parameters are listed in Table 1. As above, two equally valid interpretations exist for this dataset; it is of note that this was not identified in our original study. Solution 2 is approximately that given in Rapatskiy et al.²⁹

4.1.4 W-band ^{17}O -HYSCORE. To address the remaining ambiguity in assigning the relative signs of the A_{iso} and D terms, we turned to ESEEM, namely, 2D-ESEEM techniques such as HYSCORE.⁷⁰ As compared to EDNMR, this technique has intrinsic advantages including enhanced linewidth resolution. Furthermore, HYSCORE transparently isolates the two anisotropic contributions to the spectral pattern, namely the hyperfine anisotropy and quadrupole interaction. Hyperfine anisotropy broadens along the anti-diagonal whereas the quadrupole interaction broadens along the diagonal.^{26,32} In this instance however it is the resolution of multiple correlation ridges that allows a definite assignment of the relative signs of the two hyperfine tensor components. Figure 5 shows HYSCORE data of the ^{17}O labeled **DTNE** complex. In the unlabelled ^{16}O **DTNE** sample two discrete cross peaks were observed in the ++ frequency quadrant, centered about the Larmor frequency of ^{14}N [$v_n(^{14}\text{N}) = 10.4$ MHz, $B_0 = 3.37$ T]. They both have a crescent structure with the maxima appearing at [5 15, 15 5] MHz. These cross peaks are consistent with a dominantly isotropic ^{14}N hyperfine coupling of ~ 10 MHz. In the corresponding ^{17}O labeled **DTNE** complex, two new signals are observed: a structured correlation ridge centered about the Larmor frequency of ^{17}O [$v_n(^{17}\text{O}) = 19.4$ MHz, $B_0 = 3.37$ T] and a second ridge which

Figure 5. **A)** W-Band ^{17}O -HYSCORE of the $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ DTNE complex measured at the center field 3.372 T. The spectrum is an average of three τ values (396, 408 and 420 ns) and is symmetrized about the diagonal. Raw data shown in the **supp. info S3**. **B,C)** A simulation showing the 1st and 2nd ^{17}O correlation ridges assuming an axial hyperfine tensor. In panels **B** and **C** the A_{iso} was fixed as either negative or positive respectively, assuming a negative anisotropic/dipolar hyperfine term (A_{aniso} or D).

Table 1. Fitted spin Hamiltonian hyperfine tensor parameters for all Mn model systems; comparison to BS-DFT

		^{17}O bridge hyperfine tensor $ A_i $ /MHz								
		Experiment							BS-DFT	unique axis
			A_1	A_2	A_3	A_{iso}	D	η	$A_{\text{iso}}(\text{av.})^a$	
DTNE	$\text{Mn}^{\text{III}}\text{-}\mu(\text{O})_2\text{-Mn}^{\text{IV}}$	1	16.9	11.5	-12.5	+5.3	-8.9	0.3	+5.2	z
		2	16.2	2.9	9.0	+9.4	+3.4	0.9		x
BIPY	$\text{Mn}^{\text{III}}\text{-}\mu(\text{O})_2\text{-Mn}^{\text{IV}}$	1	17.1	11.5	-13.4	+6.4	-8.9	0.2	+8.2	z
		2	17.0	2.2	7.8	+9.0	+4.0	0.7		x
MnCat	$\text{Mn}^{\text{III}}\text{-}\mu(\text{O})_2\text{-Mn}^{\text{IV}}$		18.1	7.5	-10.0	+5.2	-7.6	0.3	+6.7 to +7.9 ^b	z
R2lox	$\text{Fe}^{\text{III}}\text{-}\mu(\text{OH})\text{-Mn}^{\text{III}}$		17.0	13.0	33.0	+21.0	+6.0	0.3	-21.6 ^c	z

^a) average calculated value for the two oxygen bridges using summation method i.e. approach (iii), see Table 2.

^b) average calculated value for the two oxygen bridges, dependent on assignment of Mn^{IV} site

^c) projected using the averaging method

appears about 1.5 times the Larmor frequency (29.3 MHz). The latter ridge is distorted due to the limited microwave excitation bandwidth (≈ 30 MHz). When measured at single τ values, the 1st correlation ridge resolves approximately 6 peaks spaced by about 2 MHz (**supp. info. S3**). These represent blind spot artifacts that are partially suppressed by averaging spectra at a series of τ values. The spectrum shown in **Fig. 5A** is an average of three τ values (396, 408 and 420 ns). The two structured ridges observed for the ^{17}O DTNE complex are derived from the single-single quantum transition and single-double quantum transition correlations within the

^{17}O sub-manifold. As two correlation ridges are observed in the HYSCORE spectrum, an estimate of both the A_{iso} and D terms can be made by inspection. D must clearly be large as there is no separation of the 1st correlation ridge; for comparison see the background ^{14}N signal (**Fig. 5A**). However, A_{iso} must also be large to account for the overlap of the 2nd correlation ridge about the diagonal; although a large A_{aniso} could also explain this overlap (in the instance where A_{iso} is zero), the corresponding 1st correlation ridge would be significantly too broad. Thus, A_{iso} and D must be of approximately the same size to reproduce the pattern. An explicit solution exists for

the width and curvature of the 1st correlation ridge of a $I = 1/2$ nucleus assuming an axial hyperfine tensor: see Eqs. 20-21. The total width of the ridge ($\Delta\nu_\alpha$) represents the largest hyperfine tensor component, whereas the curvature is given by the ratio of the dipolar component to the nuclear Larmor frequency ($\Delta\nu_\beta$).

$$\Delta\nu_\alpha = |A_{iso} + 2D| \quad (\text{Eq. 20})$$

$$\Delta\nu_\beta = \frac{9D^2}{32|\nu_n|} \quad (\text{Eq. 21})$$

Two solutions exist depending on the relative sign of the isotropic component (A_{iso}) to the anisotropic/dipolar term (D). However, calculated correlation maps shown in **Fig. 5B** and **C** show that only the solution where A_{iso} is positive (assuming D is negative) is valid as only this solution reproduces the curvature/diagonal crossing point of the 1st correlation ridge. Using this constraint, Eq. 14 and 15 yield estimates for $A_{iso} = 4$ MHz and $D = -8.5$ MHz. These parameters are in good agreement with solution 1 simulation of the EDNMR dataset. The HYSORE data presented in **Fig. 5** however does not constrain the non-axiality of the hyperfine tensor, and the inclusion of tensor rhombicity does influence estimates for the magnitude of A_{iso} and D . As shown in the **supp. info S4** a hyperfine rhombicity of $\eta = 0.5$ is consistent with the structure of both correlation ridges. It is estimated that the inclusion of rhombicity leads to an uncertainty in A_{iso} and D values of ± 1 MHz. As a final comment it is noted that the structure of the 1st correlation ridge along the diagonal should provide an estimate of the quadrupole coupling. As no structure is observed, the quadrupole coupling must be

small and is constrained by the linewidth, i.e. $|e^2qQ/h| < 2$ MHz, see **supp. info. S4**.

4.1.5 ^{17}O -EDNMR of the Mn catalase, a $\text{Mn}^{\text{III}}-(\mu\text{-O})_2\text{-Mn}^{\text{IV}}$ complex. X-ray structures exist for the manganese catalase (**MnCat**) enzyme isolated and purified from two organisms, *Lactobacillus plantarum*³ and the species used in this study, *Thermus thermophilus*.⁵ In both structures, each Mn ion has one terminal histidine and one glutamate ligand, which acts as a bidentate ligand for one Mn site and a monodentate ligand for the second Mn site, with the last coordination site filled by a water molecule. The two Mn ions are bridged by a μ -carboxylato group of a glutamate and two oxygen bridges, one of which is derived from the substrate H_2O_2 . During its catalytic cycle the **MnCat** moves between two net oxidation states: $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ and $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$. In the $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ state the two oxygen bridges are assigned as a μ -oxo/ μ -hydroxo linkage. It is possible to chemically oxidize the **MnCat** by one electron to generate the $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ oxidation state, termed the superoxidized state. This state is EPR active ($S_T = 1/2$), displaying a multiline EPR spectrum centered at $g = 2$, similar to the model complexes discussed above. Of the two, its structure is akin to the **DTNE** complex, with bis- μ -oxo, μ -carboxylato linkage between the two Mn sites. It has been previously demonstrated that oxygen atoms of the bis- μ -oxo bridge(s) and terminal water ligand of the **MnCat** exchange with ^{17}O labeled water. The rate of exchange of the terminal water ligand is rapid (< 5 s),²⁴ whereas the exchange of the μ -oxo bridge occurs much more slowly (≈ 2 hours at room temperature).²⁴ As the characterization of the oxo-bridge was target of this study, we performed a relatively slow isotope exchange procedure.

Figure 6. A) W-band EPR spectra of **MnCat** suspended in ^{17}O labeled water. B) Corresponding ^{17}O -EDNMR spectra for of the **MnCat** as a function of incubation time. C) Corresponding τ -averaged Mims and Davies ^{17}O ENDOR measured at the center field after 24 hour incubation in H_2^{17}O . Back solid lines represent the unlabeled **MnCat**, blue lines the ^{17}O -labeled **MnCat**. Red dashed lines represent a spin Hamiltonian simulation (see **Table 1**, **supp. info S6**). The different components of the simulation are shown by solid purple and green lines.

The **MnCat** was re-suspended five times in ^{17}O labeled water at room temperature, with the total procedure taking in excess of an hour. To ensure complete exchange had occurred, samples were subsequently incubated for 24 hours at room temperature.

Figure 6A shows a comparison of W-band EPR spectra recorded for the unlabeled and ^{17}O labeled **MnCat**. Line broadening is more difficult to resolve as compared to the models, in part because the intrinsic EPR linewidth is broader, see also refs.^{23,24} The corresponding EDNMR spectrum resolves a broad envelope of ≈ 10 MHz. The shape of the signal is reminiscent of the ^{17}O signal profile seen in PSII treated with ammonia. The envelope was measured as a function of the amplitude of the pump pulse (power dependence) to assess whether it represented 1 or 2 exchanged water ligands. At high pump amplitudes the center of the pattern is enhanced, indicating the profile is made up of a narrow, more weakly coupled species and a broader, more strongly coupled species, similar to that seen for the model complexes described above. The former signal is instead assigned to the exchanged water ligand. As a further check, the EDNMR profile was measured for samples which did not receive a 24 hour incubation in H_2^{17}O buffer. As expected, the narrow component of the EDNMR profile is enhanced in these samples (**Fig. 6B**). The EDNMR profile measured is nominally consistent with the earlier Q-band ENDOR study of McConnell et al.²⁴, see also section 4.18.

The putative exchangeable bridge signal, i.e. the broad envelope, was further characterized by measuring the complete 2D EDNMR surface. This dataset is again very similar to that seen for the two model complex systems described above. A spin Hamiltonian simulation constrained such that the sign of A_{iso} is positive relative to the D , value is consistent with this interpretation (**supp. info. S6**). As a final step, both Mims and Davies W-band ^{17}O -ENDOR were attempted to improve the resolution of the spectral profile (**Fig. 6C**). The Davies ENDOR spectrum is of approximately the same width as the EDNMR envelope and thus is assigned to the exchanged bridge species; note the loss of signal intensity in the center of the profile is due to blind spotting, with the width of this central hole proportional to the inverse of the preparation/inversion pulse. In contrast the τ -averaged Mims ENDOR profile selectively picks out the narrow component of the EDNMR profile, assigned to the water ligand. In the ENDOR experiment a splitting is resolved (≈ 1.5 MHz), and the line-shape, which appears to be Lorentzian, is better visualized. The Davies ENDOR spectrum can be fit using the same spin Hamiltonian parameters inferred for the exchanged μ -oxo bridge signal. The same species also explains the wings of the Mims ENDOR profile, which extend to ± 7 -8 MHz. It cannot however explain the central component. No constrained fit can be achieved. However, if approximately the same parameters are assumed as for the water ligand of ferric heme iron,⁷¹ a good fit of the data is obtained. The characteristic feature of this fit is the inclusion of a larger quadrupole coupling ($Q = 8$ MHz, $\eta = 1$), much larger than inferred for the μ -oxo bridge, which give the spectrum its characteristic Lorentzian line-shape. A large quadrupole coupling has also been measured for ^{17}O labeled water bound to Gd^{3+} .²⁷

4.1.6 ^{17}O -EDNMR of the Mn/Fe cofactor of R2lox, a $\text{Mn}^{\text{III}}-(\mu\text{-OH})\text{-Fe}^{\text{III}}$ complex. Heterobimetallic Mn/Fe cofactors represent a new paradigm in enzymology.¹⁰ One such example is the cofactor found in the small subunit (R2) of the ribonucleotide reductase class Ic (R2c).^{6,8,9} It is activated by O_2 , forming an oxygen-bridged

cofactor which serves as an in situ $1e^-$ oxidant, initiating the enzymatic reaction. We have recently characterized a homologue of the R2c cofactor, termed **R2lox**.^{9,72,73} Although its precise biological function is not yet known it is thought to catalyze oxidative chemistry, hence its name, R2-like ligand binding oxidase. Unlike R2c,⁷⁴ there exists a crystal structure of the O_2 -activated heterobimetallic cofactor in a homogeneous state.⁹ Each site has one terminal histidine ligand and the two metals are bridged by the μ -carboxylato group of one glutamate residues and one fatty acid ligand. The ligand field of the Fe ion is completed by two monodentate glutamate residues, whereas the Mn ion has a monodentate glutamate and a water ligand, which sits along the Mn^{III} Jahn-Teller axis. An oxygen from the O_2 is subsumed into the complex as μ -hydroxo bridge; its protonation state was recently confirmed using Q-band ^2H -HYSCORE.²² As with the **MnCat**, the aim here is to characterize the oxygen bridge, i.e. μ -hydroxo.

As the μ -OH bridge is much more labile than the μ -oxo bridges of **MnCat**, complete labeling of the **R2lox** could be more easily achieved without long incubations in H_2^{17}O . Background EDNMR data for the **R2lox** cofactor resolve a ^{14}N signal, previously characterized by W-band three pulse ESEEM.²² The signal is obtained under cancellation conditions, i.e. $A = 2\nu_N$, and as such, only the ν^+ line is observed, (ν^- appears at 0 MHz, inside the blind zone). Double quantum transitions are also observed for this species at ≈ 34 MHz. Spin Hamiltonian simulations of this dataset (see **supp. info S6**), using published parameters, reproduce the 2D-EDNMR profile, and thus serve as a means to validate subsequent simulations of the ^{17}O profile.

Upon ^{17}O buffer exchange, two new signal envelopes are resolved in the EDNMR spectrum, see **Fig. 7**. Unlike all earlier data, the hyperfine coupling is sufficiently large that a splitting (doublet structure) is observed. The two peaks are split by about 20 MHz, requiring that the isotropic hyperfine coupling of this species is large, and thus the bridge carries significant spin density. The same result was inferred from the large $^1\text{H}/^2\text{H}$ hyperfine coupling assigned to the μ -OH bridge i.e. $A_{\text{iso}}(^1\text{H}) = 10$ MHz.²² The signal also displays a strong field dependence demonstrating that the D term is also large, as seen for Mn/Mn dimer models discussed above. In contrast to the bridge, the water ligand is not readily observed. It is speculated though that the smaller splitting seen for the EDNMR spectrum measured on the low field edge ($A \approx 10$ MHz) may represent this species. At the other two field positions the bridge hyperfine strongly overlaps with this region, masking these transitions.

4.1.7 μ -oxo- ^{17}O model complex data, comparison to literature. The above sections demonstrate that the observed EPR line broadening seen upon ^{17}O bridge labeling systematically overestimates the isotropic hyperfine coupling. The value determined using this methodology instead best matches the largest component of the ^{17}O hyperfine tensor: i.e. $A_{\text{iso}} + 2D$. Similarly, low field ENDOR does not provide a robust description of the ^{17}O bridge hyperfine tensor for these systems. Owing to the small g -anisotropy and large ^{55}Mn hyperfine, orientation selective measurements at low fields cannot be implemented. As such, ENDOR measurements at these frequencies, be it X- or Q-band, resolve only broad featureless spectra which are not readily interpretable. This explains why the earlier studies which sought to characterize the μ -oxo bridges of Mn complexes²³ are at variance with the results presented here. In both Usov et al.²³ and McConnell et al.²⁴ the approach that was taken was to attempt

Figure 7. **A**) 2D-EDNMR spectra of the ^{17}O labeled **R2lox** cofactor. **B**) A comparison of the EDNMR signal seen for the ^{17}O labeled cofactor (blue lines) compared to unlabeled cofactor (black lines) at three field positions **I**, **II**, **III**, marked at the top of the left panel.

match the ^{17}O hyperfine coupling estimate from EPR linewidth broadening with that inferred from Q-band ENDOR. In both studies a broad, structure-less ^{17}O ENDOR signal was observed centered at 13.5 MHz which could be interpreted to match the line-broadening estimate of $A_{\text{iso}} \approx 13$ MHz. We suspect though that the ENDOR profile as measured in these earlier studies does not represent a true average coupling, but instead emphasizes the largest component of the hyperfine tensor. A pictorial demonstration of how this could occur is shown in **Fig. 8**. Due to non-linearity of the B_2 (RF) field we suspect that the Q-band signal envelope below 7 MHz i.e. the $v(^{17}\text{O})$ and $v(^{14}\text{N})$ branches are strongly suppressed. The only resolved component is a broad featureless line extending to 15 MHz (**Fig. 8B**). Its interpretation is further hampered by the overlapping ^{14}N signal which is suspected to coincide with one of the major turning points of the $v(^{17}\text{O})$ signal branch. It can also be seen in **Fig. 8C** that at frequencies lower than Q-band (X-band), all spectral components strongly overlap making any interpretation difficult.

4.1.8 Experimental summary – a magnetic fingerprint for an oxygen bridge. From the measurements described above, a magnetic fingerprint for the μ -oxo bridge of Mn complexes can be developed. In these systems the sign of the isotropic hyperfine (relative to D) is positive, of magnitude 5-10 MHz. Terminal ligand hyperfine couplings (^{14}N etc) for Mn^{III} and Mn^{IV} model complexes typically fall in the range of 2-4 MHz, with the exception of ligands along the Jahn-Teller axis of Mn^{III} which instead are approximately 12 MHz. Thus μ -oxo bridge hyperfine couplings fall between these two ranges, and can be termed intermediate. Interestingly, the magnitude of the anisotropic hyperfine is larger than that of the isotropic term. This is not the case for terminal ligands where the

hyperfine anisotropy can usually be approximated in terms of the magnetic dipolar interaction between the ligand nucleus and the electron spin. The through space magnetic dipole estimate for the ^{17}O bridge of the **DTNE** and **BIPY** complex is $D \approx 4.5$ MHz, two fold smaller than that observed experimentally i.e. 9 MHz (see **supp. info. S7**). The experimental D value is, however, of approximately the same magnitude as that reported for the oxygen bridge of the $\text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}$ cofactor of class 1a ribonucleotide reductase, i.e. $D = 8$ MHz, and the relative signs of A_{iso} and D also match this previous study.¹² The only difference is in the magnitude of A_{iso} . For the di-Fe cofactor A_{iso} is much larger ($A = 15$ MHz) presumably because $\text{Fe}^{\text{III/IV}}$ delocalizes its valence d-electrons across its ligands to a greater extent than Mn (see section 4.2.4). Similarly, the orientation of the bridge hyperfine tensor relative to the g -axis and its axial nature are also not consistent with a through space dipolar interaction explaining the hyperfine anisotropy.

The unique g -tensor axis (g_z), which for bis- μ -oxo Mn complexes is the smallest value, is typically perpendicular the plane defined by the $\text{Mn}-(\mu\text{O})_2\text{-Mn}$ atoms, with g_x and g_y instead lying within the plane. This result can be readily understood. The octahedral Mn^{IV} ion of the spin system has a high spin d^3 local electronic configuration. This electronic state is approximately spherically symmetric leading to this ion exhibiting isotropic site spin Hamiltonian properties (g , a etc). In contrast the octahedral Mn^{III} ion of the spin system has a high spin d^4 local electronic configuration. This electronic state is stabilized by a Jahn-Teller distortion, breaking the symmetry of the site. The distortion is typically along the ligand axis perpendicular to the μ -oxo plane and thus this defines the unique g (and hyperfine) tensor axis of both the Mn^{III} ion itself and of the whole complex. As the unique g -tensor axis coincides with the Jahn-Teller axis of the Mn^{III} ion it can be identified in the crystal

for both the **BIPY** and **DTNE** complexes.^{17,68} Consistent with this picture, the unique hyperfine axis of the nitrogen ligand (N1) coincides with g_z . In the **MnCat** and **R2lox** systems the unique axis is expected to lie along the Mn-OH₂ bond.

As described earlier,^{14,24,75} the magnetic dipole tensor component of a bridging ligand consists of contributions from the hyperfine coupling of the ligand to each metal site, with the contribution of each weighted by the spin projection coefficient for each metal site.^{14,24,75} The unique axis for the site hyperfine for each oxygen bridge should lie along the Mn-O bond. Assuming a $\approx 90^\circ$ Mn-O-Mn bond, summing the two site hyperfine tensors yields a projected ¹⁷O hyperfine tensor that has approximately rhombic symmetry ($\eta = 0.6$). Its unique axis should lie in the plane that is defined by the Mn-O₂-Mn bridging motif with its middle component, in signed magnitude, perpendicular to the Mn-O₂-Mn plane. The exact opposite is seen for the experimental data, where the unique axis instead coincides with g_z i.e. is perpendicular to the Mn-(μ O)₂-Mn plane. This observation suggests that, if indeed the net hyperfine coupling can be decomposed into two onsite interactions, the two site contributions are approximately equal and should be added without consideration of the sign of the local spin projections.

Interestingly the principal axis orientation of the bridge hyperfine tensor appears consistent with assignment that seen for Fe^{III}Fe^{IV} cofactor of class 1a ribonucleotide reductase.¹² In this earlier study it was observed that the unique hyperfine axis for the bridge coincides with the unique hyperfine axis of the terminal water ligand; the latter should sit orthogonal to the oxygen bridge plane.

Figure 8. A pictorial representation of the ¹⁷O (μ -oxo) and ¹⁴N (on the Jahn-Teller of axis Mn^{III}) ENDOR signals seen for the **BIPY** complex at three microwave frequencies: **A**) 9 (X-band); **B**) 34 (Q-band); and **C**) 94 GHz (W-band).

4.2 DFT Calculations

The set of model complexes principally investigated in this study (see **Fig. 1**) are all geometrically and electronically similar. Spin Hamiltonian parameters for the transition metal centers are provided in **supp. info S8**. Antiferromagnetic exchange-coupling between the two metal sites is facilitated by the overlap of a set of magnetic orbitals which reside largely on the metal centers and the p-orbitals of the μ -oxo/hydroxo bridge(s) (**supp. info. S10**).^{62,76} As has been noted in the literature, the Mn^{III} spin center has a large Jahn-Teller elongation along one axis which is orthogonal to the plane formed by the metal centers and the μ -oxo or μ -hydroxo bridge(s).^{64,76} In terms of their bridging ligand motif the set of complexes fall into four categories:

- bis- μ -oxo (**BIPY**);
- bis- μ -oxo, μ -carboxylato (**DTNE** and Mn^{III}Mn^{IV}**MnCat**);
- μ -hydroxo, di- μ -carboxylato bridges (**PivO**, **PivOH** and **R2lox**);
- mono/di- μ -hydroxo (Mn^{II}Mn^{III}**MnCat**)

The same complexes can equally be grouped into two isoelectronic sets: the set of Mn^{III}Mn^{IV} complexes (i-ii), and set of Mn^{II}Mn^{III} complexes (iii-iv), including **R2lox** which is isoelectronic to Mn^{II}Mn^{III} being a Mn^{III}Fe^{III} complex. As will be shown below, neither of these groupings are appropriate for describing trends seen in the calculated bridge hyperfine coupling. Instead it is the simpler criterion, namely the protonation state of the bridge (oxo vs. hydroxo), that is the principal factor in determining the magnitude of the calculated ¹⁷O hyperfine coupling (see **Tables 1** and **2**).

4.2.1 BS-DFT estimates for the hyperfine constant of μ -oxo bridge(s) of synthetic model complexes. As described in the Theory section, there is no consensus methodology using BS-DFT for the calculation of ligand hyperfines which involve more than one spin carrying fragment (metal center). Here we examine three approaches, all of which should be considered as largely empirical: i) using the raw bridge hyperfine without correction; ii) projecting the raw hyperfine for each spin fragment the bridge ligates and averaging the result; and iii) projecting the raw hyperfine for each spin fragment the bridge ligates and summing the values. The results appear in Table 2. Before comparing the three methods it should be noted that the raw calculated hyperfines are similar for all bis- μ -oxo bridged Mn dimers (6.1-9.8 MHz), suggesting that BS-DFT can be used to discriminate this type of species, even if the absolute value is uncertain.

The raw hyperfine though seems to systematically overestimate the measured hyperfine, in some cases by a factor of two. In contrast, averaging the two projected hyperfine coupling systematically underestimates the measured hyperfine, by up to a factor of two. The third method yields the best results, but this also has a tendency to overestimate the hyperfine coupling. Agreement between experiment and theory is particularly good for the **DTNE** complex, the best characterized of the set of three models, and poorest for the catalase, for which bridge estimates are less robust owing to the overlapping water ligand signal and the inequivalence and likely incomplete exchange of the two bridging oxygen atoms.

Table 2. Calculated BS-DFT ^{17}O bridge hyperfine coupling constants for Mn model complexes

^{17}O bridge hyperfine tensor /MHz											
		Exp.	Raw BS-DFT		Site, a_k		Average		Projected		
			a_k^{BS}	% error	Mn ^{III}	Mn ^{IV}	A_k	% error	A_k	% error	
DTNE	1	+5.3	+6.1	+18.1	+1.5	-2.1	+2.6	-52	+5.1	-4	
	2		+6.3	+20.6	+1.6	-2.1	+2.6	-51	+5.2	-2	
BIPY	1	+6.4	+9.8	+53.8	+2.5	-3.3	+4.1	-36	+8.2	+28	
	2		+9.7	+52.2	+2.4	-3.3	+4.1	-37	+8.1	+27	
MnCat (A)	1	+5.2	+9.8	+88.5	+2.4	-3.3	+4.1	-21	+8.2	+58	
	2		+8.0	+53.8	+2.0	-2.7	+3.4	-40	+6.7	+29	
MnCat (B)	1		+8.8	+69.2	+2.2	-2.9	+3.7	-29	+7.3	+41	
	2		+7.4	+42.3	+2.2	-3.0	+3.1	-40	+7.5	+44	
PivO	-	-	11.1	-	2.2	2.8	-4.4	-	-8.9	-	

Good agreement for the summation method may, to some extent, be rationalized in the context of terminal ligand hyperfine estimates. For the set of Mn^{III}Mn^{IV} model complexes it has been observed that the magnitude of the isotropic hyperfine coupling constants is significantly different for the set of 1st coordination sphere ligands. Ligand hyperfines broadly fall into three categories: i) axial ligands of the Mn^{III} ion which lie along the Jahn-Teller axis; ii) terminal equatorial ligand of the Mn^{III} ion and all terminal ligands of the Mn^{IV} ion; and iii) bridging ligands, which represent equatorial ligands of both the Mn^{III} and Mn^{IV} ion. Category (i) has the largest hyperfine coupling constants, greater than 10 MHz whereas category (ii) is 5-10 fold smaller. Such observations have been made both experimentally and computationally. As has been shown in the previous experimental sections, bridging hyperfine coupling constants (category iii) fall in between these two ranges. The simple projection method described in the Theory section readily rationalizes this result. As seen from Eq. 7-19, the magnitude of the bridge hyperfine coupling constant is the weighed sum of the on-site hyperfine from each Mn. As such, it should be approximately twice that seen for corresponding terminal equatorial ligands, which only have one contribution. For example, for the BIPY model the individual calculated on-site hyperfine couplings for the Mn^{III} and Mn^{IV} spin fragments are 4.8 and 3.2 MHz, respectively, yielding an overall coupling of 8.0 MHz. By way of comparison the averaging method, by design, will yield a bridge estimate that is of the same magnitude as for a terminal ligand, i.e. 4 MHz. Additionally it is important to note that the hyperfine coupling constants for ^{17}O ligands should be approximately 2 fold larger than those calculated for the ^{14}N ligands, as the Fermi contact term scales with the nuclear g-value (Eq. 22):

$$a_{\text{iso}} = \frac{4\pi}{3} \frac{g g_n \beta \beta_n}{\langle S_z \rangle} \rho(\bar{R}_n) \quad \text{Eq. 22}$$

where g , g_n , β and β_n are as defined in the theory section and ρ is the spin density at the nucleus. The ratio of g_n for ^{17}O and ^{14}N is -1.88. Thus, as a back of the envelope calculation, assuming an equivalent spin population for a bridging ^{14}N in place of the μ -oxo bridge the expected contributions from Mn^{III} and Mn^{IV} would be -2.5 and -1.7 MHz. Both values are within the range seen for termi-

nal equatorial nitrogen ligands coordinated to Mn^{III} or Mn^{IV} spin centers.⁶²

The influence of the implicit solvation (COSMO) dielectric constant was also tested. Calculations on a DTNE Mn^{III}Mn^{IV} complex without methyl groups on the DTNE ligand were completed with varying values of the dielectric constant ranging from zero to 80. Additionally, a completely conductive solvent was also tested ($\epsilon = \infty$). The calculated results indicate that there is a small, less than 0.5 MHz change, with respect to the implicit solvent model chosen (see **supp. info S9**). These results imply that the choice of a dielectric constant will only minimally impact the results of protein cluster models. The effect of explicit solvation is beyond the scope of this work.

4.2.2 BS-DFT estimates for the hyperfine constant of μ -oxo bridges and the water ligand of MnCat poised in the superoxidized state. The same good agreement is seen for the summation method for the super-oxidized state of the MnCat. For this system there is some ambiguity however with regard to assignment of local oxidation states to +III or +IV. Both options were tested, termed here Catalase model A and Catalase model B. The key difference between the two models is whether the H₂O ligand is coordinated to the Mn^{III} (model A) or Mn^{IV} (model B). As can be seen on Table 2 the calculated values for the superoxidized state of MnCat fall within the same range as those of the model complexes DTNE and BIPY. Interestingly, but perhaps not surprisingly, the two different models do not allow for the absolute oxidation states of the Mn centers to be determined.

The assignment of the Mn oxidation states is far more important when considering the estimate for the ^{17}O hyperfine of the H₂O ligand. ^1H -ENDOR resolves large hyperfine coupling for exchangeable hydrogens ≈ 17 MHz. Such couplings have been interpreted in terms of the water ligand being associated with the Mn ion that carries majority spin ($\rho = 2$) i.e. the Mn^{III} ion, and subsequent inhibition studies support this assignment. Similarly, a theoretical study of Sinnecker et al.⁷⁷ supported the Mn^{III} being the the 5-coordinate Mn with its open coordination site filled with water. If this is the case though, comparison to the model complexes above would suggest that the water ligand is the axial ligand of the Mn^{III} ion on the Jahn-Teller axis. Calculations of the ^{17}O hyperfine of this terminal water ligand place it at about -20.5 vs. +1.5 MHz for

Table 3. Calculated BS-DFT hyperfine coupling constants for the R2lox and related model systems

	¹⁷ O hyperfine tensor /MHz							
	Raw BS-DFT a_k^{BS}		Site, a_k			Projected, A_k		Average, A_k
	H ₂ O	bridge	H ₂ O	bridge	H ₂ O	H ₂ O	bridge	
				Fe ^{III} (Mn ^{II})	Mn ^{III}			
R2lox Mn-(μOH)-Fe	42.9	-53.9	-10.7	-10.8	13.5	14.3	-43.2	-21.6
PivOH Mn/Fe	-	-58.5	-	-11.7	14.6	-	-46.8	-23.4
PivOH Mn/Mn	-	-34.8	-	-7.0	8.7	-	-27.8	-13.9
MnCat Mn/Mn	-28.5	-24.5	-5.7	-4.9	6.1	-13.3	-19.6	-9.8
II,III		-34.8		-7.0	8.7		-27.9	-13.9
MnCat Mn/Mn	28.9	-24.7	-7.2	-5.0	6.2	9.6	-19.8	-9.9
III,II		-38.0		-7.6	9.5		-22.3	-15.2

models Catalase A and Catalase B, respectively. Notably the calculated hyperfine for the water occupying the axial position is significantly larger than results reported here and for the aforementioned proton/¹⁷O ENDOR study. As such the experimental results and interpretation do not agree with the calculated values, a discrepancy which is still undergoing investigation.

4.2.3 The bis-μ-oxo vs. mono-μ-oxo bridging motif. Lower valence Mn complexes have a tendency to stabilize mono-μ-oxo bridge motif instead of a bis-μ-oxo motif.⁷⁸ Although measurements were attempted for these systems, the more liable nature of the Mn^{II} ion in aqueous solution led to complex decomposition during labeling. Nevertheless, these systems could be examined *in silico*. Table 2 lists calculated ligand hyperfine coupling for the Mn^{II}/Mn^{III} **PivO**. Terminal nitrogen hyperfine coupling for these complexes fall into the same two classes described above for Mn^{III}/Mn^{IV} complexes. Interestingly, the calculated bridge hyperfine for the mono-μ-oxo bridge is within the range seen for the higher valence Mn^{III}/Mn^{IV} bis-μ-oxo bridge complexes. This is somewhat unexpected, but not truly surprising as the exchange pathway between the two Mn ions is qualitatively similar to those of the bis-μ-oxo complexes; comprising mostly of combinations of ligand p-orbitals and along with metal d-orbitals (**supp. info. S10**). Thus the general rule of thumb outlined above appears to be robust towards the precise structure of the bridge with the bridge hyperfine being approximately two fold larger than a terminal nitrogen ligand (accounting for the nuclear g-value), and smaller than that seen for ligands along the Jahn-Teller axis of the Mn^{III} ion.

4.2.4 The effect of protonation of the bridge. Although not experimentally accessible for the same reasons as the Mn^{II}Mn^{III} **PivO** complex, BS-DFT calculations were performed on the mono-μ-hydroxo bridged **PivOH** complex. Here, unlike the complexes presented previously the μ-hydroxo bridge has a much larger (27 vs. 9 MHz) calculated hyperfine coupling constant upon the protonation of the μ-oxo bridge. This result is somewhat counter intuitive as the protonation of the bridge should result in a lowered covalency of the Mn-oxo bond. However, protonation of the μ-oxo bridge simultaneously leads to an increase in s-character of the Mn-oxo bonds (**supp. info. S10**) resulting in a larger core spin-polarization which in turn leads to larger isotropic hyperfine values (via the Fermi contact term). Interestingly the Mulliken spin populations show the expected intuitive trend with spin populations of

0.10 and -0.02 for the oxygen atoms of the μ-oxo and μ-hydroxo bridges, respectively. It should be noted, however, that such a spin population analysis, for either the ligand has been shown to not correlate with the actual calculated isotropic hyperfine couplings, which is only representative of the core spin polarization (Eq. 22); and not the total unpaired spin density of the EPR active nuclei.⁷⁹ Similar results are seen in the bis-μ-hydroxo bridged Mn^{II}Mn^{III} catalase models which further supports the notion that the bridge hyperfine coupling should increase upon bridge protonation, specifically that the experimental hyperfine of μ-hydroxo bridges should be approximately 2 fold larger; note this estimate takes into account the expected overestimation of the calculated hyperfine couplings (see **Table 2**).

4.2.5 BS-DFT estimates for the hyperfine constant of the μ-hydroxo bridge and water ligand of R2lox. Calculated hyperfine coupling constants for the cluster model of R2lox are presented in **Table 3**. **R2lox** itself presents an interesting deviation from the other model systems studied in that it represents a heterometallic complex with a μ-hydroxo bridge. As stated above, it can be considered structurally and electronically similar to the Mn^{II}Mn^{III} **PivOH** complex as Fe^{III} is isoelectronic to Mn^{II}, the two metals are bridged by a μ-hydroxo ligand and the two metal ions couple together antiferromagnetically to give a ground state spin of $S = \frac{1}{2}$. As per the last section, the expectation is that the theoretical ¹⁷O hyperfine would be approximately the same as calculated for the **PivOH** complex (≈20 MHz), which would be consistent with the experimental **R2lox** data. This, however, is not the case. As can be readily seen from **Table 3**, the calculated results are approximately 2 fold larger using the summation method. Here it seems instead that the averaging model⁶⁶ is more appropriate, reproducing the experimental result i.e. 21 MHz. The same good agreement using the averaging method was seen previously by Noodleman and co-workers of the di-Fe cofactor of ribonucleotide reductase.⁶⁶ It can be further shown that this effect is solely a consequence of the R2lox cofactor containing an Fe ion. Calculations performed on the hypothetical Mn^{III}Fe^{III} **PivOH** complex where the Mn^{II} was replaced with Fe^{III}, yield essentially the same 2-fold increase in the estimated bridge hyperfine coupling as compared to the di-Mn homologue. This significant difference indicates that the Fe^{III} ion plays a different role from that of the Mn^{II} ion in the distribution of spin across the molecule. A difference of one unit of nuclear charge for the Fe vs. Mn counter intuitively results in Fe complexes having

more delocalized unpaired d-shell electrons leading to more spin polarization on the ligands coordinated to the Fe metal center. Mn, on the other hand, has much more localized d-shell electrons, as can be seen in a comparison of Mn *vs.* Fe Mulliken or Löwdin spin populations. Consequently, this means that the larger calculated bridge hyperfine coupling values seen in complexes that contain a Fe^{III} metal center can be rationalized as indicating that a significantly larger portion of the spin polarization located on the μ -hydroxo bridging ligand is derived from electronic overlap with the orbitals of the Fe^{III} ion rather than the Mn^{III} ion. Thus the fundamental assumption presented in the Theory section, namely that the bridging ligand is “shared” by both spin sites, is not met for such complexes, and therefore the summation method fails. Clearly we are not at the point where BS-DFT can provide confident predictions for bridging hyperfine coupling constants for an arbitrary system, and indeed it may be necessary to move beyond BS-DFT to develop a fully consistent methodology.

5. CONCLUSIONS AND OUTLOOK

The above study demonstrates that oxygen bridges of Mn complexes can be described in depth using multifrequency, multiresonance EPR techniques. Interestingly the experimental isotropic hyperfine coupling seen between different complexes +5.3 MHz (**DTNE**), +6.4 MHz (**BIPY**), +5.2 MHz (**MnCat**) is approximately invariant. The insensitivity of the isotropic hyperfine coupling to the exact geometry of the bridging unit, the ligand scaffold and the polarity of the local environment (solvent *vs.* protein) suggests that its magnitude can be used as a robust marker for this bridging type. This result is extended by DFT calculations which show a similar invariance in the ¹⁷O bridge hyperfine coupling value within the tolerance typically achieved for ligand hyperfine couplings. It also holds for complexes of different net Mn oxidation state, or for complexes that contain either a single or bis- μ -oxo bridging network. The only parameter that significantly changed the observed hyperfine coupling was protonation of the bridge, leading to a 2-fold increase. As stated above, this observation is counter-intuitive. As bridge protonation should lead to a decrease in covalency of the bridging oxygen the hyperfine coupling should instead decrease. It is hypothesized that this effect is due to interplay between the spin density, which is predominantly associated with the p-orbitals of the bridging oxygen, and the spin polarization as described above.

This μ -oxo *vs.* μ -hydroxo fingerprint will serve as benchmarks for expanded catalytic studies on metallocofactors. From the perspective of studying the catalysis of such Mn metallocofactors, the protonation state of the bridge is of critical importance. While the metal oxidation state etc can be assessed by measuring the metal hyperfine, the net bridge protonation state of the bridging network can be ambiguous. An example would be if the complex contains a both a deprotonated μ -oxo bridge and a singly protonated μ -hydroxo bridge. As demonstrated here ¹⁷O labelling of this hypothetical complex should provide a marker for each oxygen bridge type allowing the connectivity of the complex to be experimentally constrained. The Mn/Mn cofactor found in the ribonucleotide reductase enzyme class 1b is one such example^{7,80} which could be studied using this approach.

Of particular interest is the characterization of reaction intermediates.⁸¹ Several metal cofactors activate small molecules via μ -oxo bridge assembly. The precise fate of such reactants can thus be tracked using the methods described above. One such example is nature's water oxidizing complex, a tetramanganese-calcium cofac-

tor, found in Photosystem II. Our recent work has shown that the cofactor contains a fast exchanging oxo bridge mid-way through its catalytic cycle. This bridge is considered the most likely candidate for the 1st substrate water bound during catalysts. The identity of the exchangeable bridge has previously been assigned to O5, the oxo bridge between Mn3 and Mn4.

This assignment was based on site perturbation: addition of NH₃, which displaces a terminal water ligand of the cofactor (W1), was seen to strongly perturb the exchangeable bridge's hyperfine coupling (30% decrease). This change has been rationalized by a *trans* effect; the oxo bridge *trans* to W1 is O5. BS-DFT calculations quantitatively supported this assignment. While projected hyperfine couplings were not available at the time, it could be shown that calculated raw hyperfine coupling of the μ -oxo bridge O5 is sensitive to NH₃ binding, owing to an elongation of the Mn4-O5 bond. The BS-DFT calculations presented here suggest that the use of summation method may be appropriate for this system to calculate the true hyperfine coupling. Indeed taking raw hyperfine coupling constants from our previous study³³ and using the summation method yields estimates within 30% of experiment. The calculation of such bridging hyperfine couplings and the detection of bound substrate sites in all catalytic states of the WOC cofactor forms ongoing work of our laboratory.

ASSOCIATED CONTENT

Supporting Information

This contains additional material and is arranged as follows: S1) ¹⁷O/¹⁸O labeling of synthetic complexes as monitored by mass spectrometry; S2) Multifrequency CW and pulse EPR spectra of the **BIPY** complex; S3) Raw HYSORE data of the **DTNE** complex; S4) Additional spin Hamiltonian simulations of the W-band HYSORE data collected for the **DTNE** complex; S5) Additional EDNMR data of the **MnCat**; S6) Spin Hamiltonian simulations of the **DTNE**, **BIPY**, **MnCat** and **R2lox** 2D-EDNMR data; S7) Tabulated magnetic-dipole coupling for all structural models; S8) Additional DFT calculated parameters for models studied; S9) Effect of dielectric constant used in calculations S10) Magnetic unrestricted corresponding orbitals (UCO) for the **PivO**, **PivOH**, and the Mn^{III}Fe^{III} **PivOH** model complexes; S11) References; S12) Tabulated xyz coordinates for all structural models. The material is available free of charge via the Web at <http://pubs.acs.org>

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ABBREVIATIONS

EPR, electron paramagnetic resonance, ENDOR, electron nuclear double resonance, ELDOR, electron electron double resonance, NMR, nuclear magnetic resonance, EDNMR, ELDOR detected NMR, SQ, single quantum, DQ, double quantum.

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Table of Contents artwork