Structure, ligands and substrate coordination of the oxygen-evolving complex of photosystem II in the \( S_2 \) state: a combined EPR and DFT study†

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The \( S_2 \) state of the oxygen-evolving complex of photosystem II, which consists of a \( \text{Mn}_4\text{O}_5\text{Ca} \) cofactor, is EPR-active, typically displaying a multline signal, which arises from a ground spin state of total spin \( S_g = 1/2 \). The precise appearance of the signal varies amongst different photosynthetic species, preparation and solvent conditions/conditions. Over the past five years, using the model species \( \text{Thermosynechococcus elongatus} \), we have examined modifications that induce changes in the multline signal, i.e. \( \text{Ca}^{2+}/\text{Sr}^{2+} \)-substitution and the binding of ammonia, to ascertain how structural perturbations of the cluster are reflected in its magnetic/electronic properties. This refined analysis, which now includes high-field (\( W \)-band) data, demonstrates that the electronic structure of the \( S_2 \) state is essentially invariant to these modifications. This assessment is based on spectroscopies that examine the metal centres themselves (EPR, \( ^{55}\text{Mn}-\text{ENDOR} \)) and their first coordination sphere ligands (\( ^{14}\text{N} \) and \( ^{17}\text{O} \)-ESEEM, -HYSCORE and -EDNMR). In addition, extended quantum mechanical models from broken-symmetry DFT now reproduce all EPR, \( ^{55}\text{Mn} \) and \( ^{14}\text{N} \) experimental magnetic observables, with the inclusion of second coordination sphere ligands being crucial for accurately describing the interaction of \( \text{NH}_3 \) with the \( \text{Mn} \) tetramer. These results support a mechanism of multiline heterogeneity reported for species differences and the effect of methanol [\( \text{Biochim. Biophys. Acta, Bioenerg.} \, 2011, \, 1807, \, 829 \)], involving small changes in the magnetic connectivity of the solvent accessible outer \( \text{Mn}_{4\text{a}} \) to the cuboidal unit \( \text{Mn}_4\text{O}_5\text{Ca} \), resulting in predictable changes of the measured effective \( ^{55}\text{Mn} \) hyperfine tensors. \( \text{S}^{2\pm} \) and \( \text{NH}_3 \) replacement both affect the observed \( ^{17}\text{O} \)-EDNMR signal envelope supporting the assignment of O5 as the exchangeable \( \mu \)-oxo bridge and it acting as the first site of substrate inclusion.

1 Introduction

In oxygenic photosynthesis, light-driven water oxidation is catalysed by the oxygen-evolving complex (OEC) of the transmembrane protein complex photosystem II (PSII). The OEC consists of a \( \mu \)-oxo-bridged tetramanganese-calcium cofactor (\( \text{Mn}_4\text{O}_5\text{Ca} \)), embedded in its protein matrix. This matrix includes the redox-active tyrosine residue \( \text{Y}_{2} \) (D1-Tyr161), which couples electron transfer between the \( \text{Mn}_4\text{O}_5\text{Ca} \) cluster and P680+*, the photooxidant of the PSII reaction centre. The four-electron water oxidation reaction requires four consecutive light-induced charge separation events, driving the catalytic cycle of the OEC.1-3 This cycle involves five redox intermediates, the \( S_n \) states, where \( n = 0-4 \) indicates the number of stored oxidizing equivalents. All \( S \)-state transitions represent oxidations of the \( \text{Mn}_4\text{O}_5\text{Ca} \) cluster by \( \text{Y}_{2}^* \) with the exception of the regeneration of \( S_0 \) from \( S_4 \), which proceeds spontaneously under the release of molecular triplet oxygen and the rebinding of at least one substrate water molecule. The rate-limiting step, oxidation of \( S_1 \) by \( \text{Y}_{2}^* \), has prevented the transient, fast-decaying \( S_1 \) state from being characterized yet. For a general introduction into water oxidation by the OEC, see ref. 3-8.

The structure of the \( \text{Mn}_4\text{O}_5\text{Ca} \) inorganic core resembles a ‘distorted chair’ where the base is formed by a \( \mu \)-oxo-bridged...
conditions used, the $S_2$ state can also exhibit other broad EPR configuration gives rise to the well-known $S_2$ state EPR multiline peaks, with a line spacing around 87 G. 23 Depending on the $g$ signal centred at $g = 2.0$, with a hyperfine pattern of at least 24 frequencies, $^{31-34}$ have enabled a more detailed examination of the electronic structure by uniquely determining four $^{55}$Mn hyperfine interactions that give rise to the multiline pattern of the corresponding $S_T = 1/2$ EPR signal. This ENDOR analysis has strongly suggested that the OEC contains one Mn$^{III}$ ion and three Mn$^{IV}$ ions when poised in the $S_2$ state. $^{30-34}$

OEC models developed from broken-symmetry (BS) density functional theory (DFT) have been crucial for the interpretation of EPR and related magnetic resonance results. $^{12,14,18,31,32-44}$ These calculations now allow different S-state models to be assessed based on the spin multiplicities and relative energies of their lowest magnetic levels, and, by means of the spin projection formalism (see ref. 18 and 36), the metal and ligand hyperfine couplings. This strategy enables the assignment of individual Mn oxidation states and coordination geometries and represents a method to develop unified models of the OEC that combine constraints from X-ray diffraction, EXAFS and magnetic resonance.

Site perturbation of the OEC provides a means to further characterize the global geometric and electronic structure of the Mn tetramer and obtain information about localized changes associated with the introduced modification. The two best described OEC perturbations in the literature are (i) the replacement of the Ca$^{2+}$ with a Sr$^{2+}$ ion $^{43,45-47}$ and (ii) the binding of NH$_3$ to the cluster. $^{48-55}$ Both of these perturbations do not inhibit functional turnover of the catalyst, but do modify the kinetics of O-O bond formation, substrate water uptake and its subsequent deprotonation. A brief description of these two modified OEC forms is given below:

(i) Sr$^{2+}$ can be introduced into the OEC through chemical removal of the Ca$^{2+}$ ion $^{45,46,56}$ or alternatively by biosynthetic incorporation in cyanobacterial cultures grown in the presence of SrCl$_2$ instead of CaCl$_2$. $^{47}$ Without Ca$^{2+}$ (or Sr$^{2+}$) the OEC is inhibited $^{56-63}$ not progressing further than a modified form of the $S_2$ state, i.e. the $S_2'Y_2^*$ state. $^{60-62}$ Sr$^{2+}$ is unique as it is the only ion which can replace the Ca$^{2+}$ ion while retaining catalytic activity, albeit at a lower enzymatic rate. $^{45,47,56}$ Presumably this is because Sr$^{2+}$ has a similar size and Lewis acidity as Ca$^{2+}$. $^{64}$

While slowing the turnover rate of the catalyst, Sr$^{2+}$ substitution at the same time enhances the exchange rate with bulk water of at least one of the bound substrates. $^{65,66}$ as observed by time-resolved membrane inlet mass spectrometry (MIMS). $^{67,68}$ This behaviour suggests the Ca$^{2+}$ ion may play an important role in substrate water binding and possibly proton release (for reviews, see ref. 3 and 69).

(ii) Ammonia binding to the Mn cluster (in the presence of high Cl$^-$ concentrations) $^{48,50,51,54}$ only occurs upon formation of the $S_2$ state. It is subsequently released at some point during the S-state cycle ($S_i \rightarrow S_0 \rightarrow S_1$), such that it is not bound upon return to $S_1$. $^{51}$ As with Sr$^{2+}$ replacement, NH$_3$ binding does not inhibit catalytic function. In the higher plant electron spin echo envelope modulation (ESEEM) study of Britt et al.,$^{70}$ NH$_3$ was shown to bind as a direct ligand of the Mn tetramer. The precise binding site and coordination mode of the NH$_3$ molecule was the subject of a recent study on cyanobacterial PSII from our laboratory. $^{44}$ By employing electron electron double resonance (ELDOR)-detected NMR (EDNMR), it was concluded that NH$_3$
replaces the water ligand of MnA4 trans to the O5 bridge (W1, Fig. 1b). As the binding of NH3 was also shown not to affect substrate exchange rates, these results suggest W1 does not represent a substrate water. One or more additional NH3 binding sites, which are inhibitory, are known but are less well characterized.49,50,71

Here, we present an extension of our earlier multifrequency EPR studies31,34,44 of different ‘archetypal’ multiline forms, namely the native (Mn4O5Ca), Sr2+ substituted (Mn4O5Sr) and NH3-treated (Mn4O5Ca-NH3) S2 states, providing a comprehensive and 14N magnetic spectroscopic observables for the native and same modifications for the X-band samples as described in ref. 14. The different OEC forms represent a starting point to examine the energetics of higher S-state transitions, as they differ with experimental conditions provide more reliable spectral line shapes than before, confirming our previous general S2 state model. Improved 55Mn ENDOR experimental conditions provide more reliable spectral line shapes before, confirming our previous general S2 state model. Extended BS-DFT models are shown to reproduce all EPR, 55Mn and 14N magnetic spectroscopic observables for the native and the modified systems, a feature not achieved previously. The experimental results and calculations for 14N/15N ligands of the various S2 state forms serve to prove that the basic electronic structure is not perturbed by these modifications, a result crucial for the interpretation of concomitant perturbations of the 17O EDNMR signal envelope. This combined experimental and theoretical approach supports our qualitative model for multiline heterogeneity, demonstrating that the magnetic connectivity between the two subunits and also within the trimeric moiety governs the structure of the multiline signal.34 This basic structural template also explains the apparent orientations of the 55Mn hyperfine tensors, as inferred from spectral simulations and single crystal measurements,32 and potentially provides a framework to further examine substrate binding. The different OEC forms represent a starting point to examine the energetics of higher S-state transitions, as they differ with regard to substrate binding and the kinetics of O–O bond formation and O2 release.

2 Materials and methods

2.1 PSII sample preparation

Ca2+ and Sr2+-containing PSII core complex preparations from T. elongatus73 were isolated as reported before47,73,74 with the same modifications for the X-band samples as described in ref. 44. Universal 15N-labelling of the PSII proteins was achieved by growing the cyanobacteria in a modified BG11 or DTN medium that contained 15NH4Cl and 15NO3 salts as the sole nitrogen source.75 PSII preparation were stored at −80 °C until use. Dark-adapted samples were placed in X-, Q- or W-band quartz tubes with inner diameters of 3.0, 1.6 and 0.6 mm, respectively, and kept at 77 K (liquid N2) until use. A sample concentration of 3.0–4.0 mg chlorophyll per ml was used throughout this study. All work was conducted in the dark or under dim green light. NH4 modification was conducted as described in ref. 44, see also Section S1 of the ESI.† Isotopically enriched H218O buffer exchange was achieved as described in ref. 14.

2.2 X-, Q-, and W-band EPR and ENDOR measurements

X-band (≥ 9 GHz) continuous-wave (CW) EPR spectra were recorded at liquid He temperatures on a Bruker ELEXSYS E500 spectrometer, equipped with an ESR 900 liquid helium flow cryostat and an ITC503 helium flow temperature controller (Oxford Instruments Ltd). X-band pulse EPR measurements were carried out at 4.3 K using a Bruker ELEXSYS E580 spectrometer, equipped with a CF935 cryostat and an ITC503 temperature controller. Q-band (≥ 34 GHz) pulse EPR experiments were performed around 5 K using an ELEXSYS E580 spectrometer, equipped with a homebuilt TE011 microwave cavity,76 a CF935 liquid helium cryostat, an ITC-503 temperature controller and a radiofrequency (RF) amplifier ENI 5100L. W-band (≥ 94 GHz) EPR experiments were performed at 4.8 K using a Bruker ELEXSYS E680 EPR spectrometer. All W-band experiments were carried out using a homebuilt ENDOR microwave cavity, which contained a solenoid of Teflon coated silver wire integrated into a commercial ENDOR probe head (Bruker). In order to ensure broadband microwave excitation and minimize distortions, the loaded quality factor QL was lowered to 700 to obtain a microwave frequency bandwidth of 130 MHz.

Electron spin echo-detected (ESE) field-swept EPR spectra were measured using the pulse sequence t,p–t–4p–p–echo,77 three-pulse ESEEM spectra by use of t,p–t–p–p–echo77 and hyperfine sublevel correlation (HYSCORE) spectra by employing t,p–t–p–T1–2p–T2–4p–p–echo.78 The lengths of the π/2 microwave pulses were generally set to t = 16 ns (X-band), 12 ns (Q-band) and 24 ns (W-band), respectively. For ESE-detected EPR experiments, inter-pulse distances were τ = 260 ns (Q-band) and 300 ns (W-band). For the three-pulse ESEEM measurements, multiple τ values in the ranges τ = 136–248 ns (X-band) and 200–356 ns (Q-band) and an optimum τ = 260 ns for the HYSCORE experiments were chosen to account for blind-spotting artefacts. Q-band 55Mn-ENDOR spectra were acquired employing the Davies-type pulse sequence t,mw–T–t–p–p–T–p–p–echo80 using a length t,mw = 24 ns for the π inversion microwave pulse and a radio frequency π pulse length t,p = 3.5 μs. The length of the π/2 microwave pulse in the detection sequence was generally set to t = 12 ns and the inter-pulse delays to T = 2 μs and τ = 268 ns. A shot repetition time of 1 ms was used for all measurements. EDNMR measurements were collected using the pulse sequence t,mw–T–t–p–p–T–p–p–echo.81 The high turning angle (HTA) microwave pulse was applied at microwave frequencies ν,mw. The Hahn echo detection pulse sequence t,p–t–2p–p–echo, at a microwave frequency ν,mw matched to the cavity resonance, was set at a sufficient time T after the HTA pulse to ensure near-complete decay of the electron spin coherencies. The π/2 pulse length used for detection was t,p = 200 ns (14N, 17O) or 80 ns (15N) and an inter-pulse separation of τ = 500 ns was used. The echo was integrated ≈ 600 ns around its maximum. The spectra were acquired via continuously sweeping the HTA frequency ν,mw at a fixed magnetic field in steps of 78.1 kHz (14N), 128.9 kHz (15N) or 162.1 kHz (17O). A HTA microwave pulse of length t,HTA = 14 μs (14N, 17O) and 8 μs (15N) and an amplitude ω1 = 12–16 × 106 rad s−1 was used.
2.3 Spectral simulations

Spectra were fit assuming an effective spin $S_{\tau} = 1/2$ ground state (Section S4.2, ESIF). The basis set that describes the $^{55}\text{Mn}$ tetramer-single electron spin manifold (eqn (1)) and the $^{14}\text{N}$, $^{15}\text{N}$ and $^{17}\text{O}$ single nucleus-single electron spin manifolds (eqn (2)) can be built from the product of the eigenstates of the interacting spins:

\[
\left| \begin{array}{c}
\frac{1}{2}m_1 \frac{1}{2}m_2 \frac{1}{2}m_3 \frac{1}{2}m_4 \\
\frac{1}{2}M_{\text{m}}
\end{array} \right|
\]

(eqn (1))

\[
\left| \begin{array}{c}
\frac{1}{2}I_{\text{m}}
\end{array} \right|
\]

(eqn (2))

Here, $M$ refers to the electronic magnetic sublevel, $\pm \frac{1}{2}$; $I$ takes the values $5/2$ for $^{55}\text{Mn}$, $1$ for $^{14}\text{N}$, $\frac{1}{2}$ for $^{15}\text{N}$ and $5/2$ for $^{17}\text{O}$; $m$ takes the values $-I_m, \ldots, I_m-1, I_m$. The spin manifolds can be described by the following spin Hamiltonian:

\[
H = \beta \mathbf{B}_0 \cdot \mathbf{G} \cdot \mathbf{S} + \sum_i \left( -\frac{g_n \beta_n \mathbf{B}_i \cdot \mathbf{I} - \mathbf{S} \cdot \mathbf{A}_i \cdot \mathbf{I} + \mathbf{I} \cdot \mathbf{Q}_i \cdot \mathbf{I} \right)
\]

(eqn (3))

It contains (i) the Zeeman term for the total electronic spin, (ii) the hyperfine and (iii), except for the EPR spectra, nuclear Zeeman terms for either the metal $^{55}\text{Mn}$ nuclei, or the ligand $^{14}\text{N}$, $^{15}\text{N}$ or $^{17}\text{O}$ nuclei and (iv), for the $^{14}\text{N}$ nuclei ($I = 1$), the nuclear quadrupole interaction [NQI] term; the NQI splitting is not resolved in the $^{55}\text{Mn}$ and $^{17}\text{O}$ spectra. Spectral simulations were performed numerically using MATLAB® (R2010a, The MathWorks, Natick, MA, USA), a vector-based linear algebra package, and the EasySpin toolbox. For further information on data processing, details of the simulations and theory, see Sections S2, S3 and S4 (ESIF), respectively.

2.4 DFT calculations

All calculations were performed with ORCA. The DFT models of the OEC systems consist of 238 or 239 atoms (with $\text{H}_2\text{O}$ or $\text{NH}_3$ at the W1 position, respectively) and were constructed as described in Pantazis et al. Alternative ammonia binding modes, including terminal or bridging amido and imido substitution, can be rejected on energetic grounds alone (see Fig. S2, ESIF). Geometric optimizations of the cluster models used the BP86 density functional and the zero-order regular approximation (ZORA) and specially adapted segmented all-electron relativistically recontracted basis sets (ZORA-TZVP for Mn, O and N; ZORA-SVP for C and H). The conductor-like screening model (COSMO) was used with a dielectric constant $\epsilon = 8.0$. D3 dispersion corrections were applied in all calculations. Exchange coupling constants, assuming an isotropic Heisenberg Hamiltonian of the form

\[
\hat{H} = -2J_{ij} \sum_{i<j} \mathbf{S}_i \cdot \mathbf{S}_j
\]

and all EPR properties were computed with the TPSSh hybrid meta-GGA functional from BS-DFT calculations. The resolution of identity (RI) approximation was used in the calculation of Coulomb integrals and the chain-of-spheres approximation (COSX) was used for Hartree–Fock exchange, employing completely decontracted def2-TZVP/J auxiliary basis sets. Tight SCF convergence criteria and increased integration grids (Grid6 and GridX6) were applied throughout.

For the calculation of the hyperfine tensors, triple-zeta ZORA-recontracted basis sets were used on all atoms, while locally dense radial grids were used for Mn, N and O atoms (integration accuracy of 11 for Mn and 9 for N and O as per ORCA nomenclature). Picture change effects were applied for the calculation of EPR parameters and the complete mean-field approach was used for the spin–orbit coupling operator. The results were transformed into on-site or spin-projected values as detailed previously. To compare computed $^{55}\text{Mn}$ hyperfine coupling constants using the methods described above with experimental results, a scaling factor of 1.78 was calculated from a set of twelve Mn$^{III}$–Mn$^{IV}$ mixed-valence dimers.

3 Results and discussion

3.1 DFT models of different OEC forms in the $S_2$ state

Geometric parameters of optimized DFT cluster models of the $S_2$ state of the OEC in the $S_{\tau} = 1/2$ configuration are shown in Fig. 2 (for coordinates, see Section S5, ESIF). Four variants were considered in this study: (i) the native cofactor system ($\text{Mn}_4\text{O}_5\text{Ca}$, also see Fig. 1), (ii) the Sr$^{2+}$-substituted system obtained by replacing Ca$^{2+}$ with Sr$^{2+}$ ($\text{Mn}_4\text{O}_5\text{Sr}$), (iii) the NH$_3$-modified system obtained by replacing the H$_2$O in the W1 position with NH$_3$ ($\text{Mn}_4\text{O}_5\text{Ca}–\text{NH}_3$), and (iv) the combined Sr$^{2+}$-substituted and NH$_3$-modified system ($\text{Mn}_4\text{O}_5\text{Sr}–\text{NH}_3$). In all models, W2 was considered to be an OH$^-$ ligand, as determined previously. Mulliken spin population analysis of all four variants confirms that the only Mn$^{III}$ ion of the tetramanganese complex is Mn$_{D1}$. The three Mn$^{IV}$ ions (Mn$_{A4}$, Mn$_{B3}$ and Mn$_{C2}$) represent coordinatively saturated, 6-coordinate octahedral sites, whereas the Mn$_{D1}^{III}$ is 5-coordinate square-pyramidal, with a Jahn–Teller elongation along the axis of the Mn$_{D1}$–Asp342 carboxylate ligand, opposite to its open coordination site.

In accordance with previous DFT and QM/MM structures, the optimized Mn–Mn and Mn–Ca distances of the Mn$_4\text{O}_5\text{Ca}$ model are consistent with those determined from EXAFS spectroscopy. Only minor changes are observed between the Mn$_4\text{O}_5\text{Ca}$ and the Mn$_4\text{O}_5\text{Sr}$ models (Fig. 2). As a result of the larger radius of Sr$^{2+}$, the O–Sr bond lengths increase by 0.04 Å, while the Mn–Sr distances also increase by 0.04 Å except for Mn$_{D1}$–Sr, which is 0.03 Å shorter than the Mn$_{D1}$–Ca distance. On average, this is in line with observations from EXAFS spectroscopy and with the recent 2.1 Å resolution crystallographic model of Sr$^{2+}$-substituted PSI-I. The Mn–Mn distances are almost entirely unaffected, with the exception of Mn$_{D1}$–Mn$_{C2}$, which is shortened by 0.02 Å in the Mn$_4\text{O}_5\text{Sr}$ model.

Upon NH$_3$ substitution of W1 (Mn$_4\text{O}_5\text{Ca}–\text{NH}_3$), only the Mn$_{D1}$–Mn$_{B3}$ distance and the Ca$^{2+}$ distance from the terminal Mn ions change notably, albeit by less than 0.05 Å (Fig. 2). Only one structural element is more significantly perturbed, i.e. the position of O5, the Mn$_{A4}$–O5 distance increases by 0.05 Å with concomitant elongation along the axis of the Mn$_{D1}$–Asp342 carboxylate ligand, opposite to its open coordination site.

Abstract

In this paper, we report on the analysis of the electronic and magnetic properties of the oxygen-evolving complex (OEC) of photosystem II (PSII) using density functional theory (DFT) calculations. The OEC is a tetramanganese complex that plays a crucial role in the light-dependent oxygen evolution process in photosynthesis. Our calculations were performed on different structural models of the OEC in its $S_2$ state, with and without ammonia (NH$_3$) or strontium (Sr$^{2+}$) substitutions.

The results reveal that the Mn$^{III}$ ion is the only one that is coordinatively saturated, forming a 6-coordinate octahedral site. The other Mn$^{IV}$ ions (Mn$_{A4}$, Mn$_{B3}$, and Mn$_{C2}$) are found in 5-coordinate square-pyramidal environments. The inclusion of Sr$^{2+}$ or NH$_3$ in the model affects the structural parameters, particularly the Mn–Mn and Mn–Ca distances.

The spin–orbit coupling operator was applied, and the calculated results were transformed into on-site or spin-projected values for comparison with experimental data. A scaling factor of 1.78 was determined for the calculation of hyperfine coupling constants using the methods described above.

The calculations confirm that the only Mn$^{III}$ ion of the tetramanganese complex is Mn$_{D1}$. The three Mn$^{IV}$ ions (Mn$_{A4}$, Mn$_{B3}$, and Mn$_{C2}$) are coordinatively saturated, with 6-coordinate octahedral sites, while the Mn$_{D1}^{III}$ is 5-coordinate square-pyramidal, with a Jahn–Teller elongation along the axis of the Mn$_{D1}$–Asp342 carboxylate ligand.

The Mn–Mn distances are almost entirely unaffected by NH$_3$ substitution, except for Mn$_{D1}$–Mn$_{C2}$, which is shortened by 0.02 Å. The Mn–Sr distances increase by 0.04 Å, except for Mn$_{D1}$–Sr, which is 0.03 Å shorter than the Mn$_{D1}$–Ca distance. The results are in line with EXAFS spectroscopy observations.

The calculated hyperfine coupling constants were compared to experimental results, taking into account a scaling factor of 1.78. The calculations confirm the spin–orbit coupling operator and the complete mean-field approach used for the calculation of EPR parameters.

The structural and magnetic properties of the OEC in different states provide insights into the function of the complex in oxygen evolution and the mechanisms of charge separation in photosynthesis.
3.2 Multifrequency EPR and $^{55}\text{Mn-ENDOR}$ of the $S_2$ states of the OEC variants

Multifrequency EPR/$^{55}\text{Mn-ENDOR}$ experiments spanning the microwave range from $\approx 9$ to $\approx 90$ GHz were employed to experimentally characterize the electronic structures of the different $S_2$ state forms described above. Fig. 3A (black solid traces) depicts X-band CW EPR spectra of the $S_2$ state of native PSII ($\text{Mn}_4\text{O}_5\text{Ca}$), $\text{Sr}^{2+}$-substituted PSII ($\text{Mn}_4\text{O}_5\text{Sr}$), $\text{NH}_3$-modified (annealed) native PSII ($\text{Mn}_4\text{O}_5\text{Ca}-\text{NH}_3$) and $\text{NH}_3$-modified $\text{Sr}^{2+}$-substituted PSII ($\text{Mn}_4\text{O}_5\text{Sr}-\text{NH}_3$). Shown are light-minus-dark spectra, generated by taking the difference between the illuminated spectrum ($S_2$) and the dark-state spectrum ($S_1$) in order to remove background signals, such as from the cytochromes $b_{559}$ and $\epsilon_{550}$. The modified multiline ($\text{Mn}_4\text{O}_5\text{Sr}$, $\text{Mn}_4\text{O}_5\text{Ca}-\text{NH}_3$) displays 26 lines of altered intensity as compared to the native multiline signal with 24 lines. The $\text{Mn}_4\text{O}_5\text{Sr}-\text{NH}_3$ $S_2$ state yields essentially the same modified multiline signal; the simulation superimposing this data trace uses the parameters that fit the $\text{Mn}_4\text{O}_5\text{Sr}$ dataset.

Fig. 3B shows the corresponding W-band ESE-detected $S_2$ state multiline EPR signals of the $\text{Mn}_4\text{O}_5\text{Ca}$, $\text{Mn}_4\text{O}_5\text{Sr}$ and $\text{Mn}_4\text{O}_5\text{Sr}-\text{NH}_3$ OEC forms. Pseudo-modulated (CW-like) spectra are shown in order to more clearly visualize differences in the hyperfine structures between the three forms. Compared to earlier published data by Cox et al., the spectra are essentially free of contaminating hexaquo-$\text{Mn}^{2+}$ signals. Furthermore, there is a small difference of the centre positions of the multiline spectral envelopes, presumably due to inaccuracy in the microwave frequency calibration of this earlier study.

Fig. 3C shows the W-band ESE-detected multiline signals of the $\text{Mn}_4\text{O}_5\text{Ca}$, $\text{Mn}_4\text{O}_5\text{Sr}$, $\text{Mn}_4\text{O}_5\text{Ca}-\text{NH}_3$ and $\text{Mn}_4\text{O}_5\text{Ca}-\text{NH}_3$ $S_2$ state forms. These spectra do contain small hexaquo-$\text{Mn}^{2+}$ contaminations. This contamination manifests itself as six inverted hyperfine lines centred at $g \approx 2$, as the high-spin $\text{Mn}^{2+}$ signal ($S = 5/2$) is over-rotated when using optimal instrumental settings to visualize the $S = 1/2$ multiline signal. No $^{55}\text{Mn}$ hyperfine structure is observable in the W-band multiline EPR spectra. Thus, these signals provide no additional information on the hyperfine matrices. The utility of these high-frequency data instead is to constrain the $G$ tensor. While the spectra of the $\text{Mn}_4\text{O}_5\text{Ca}$ and the $\text{Mn}_4\text{O}_5\text{Ca}-\text{NH}_3$ cluster show similar signals, centred at $g = 1.975$, the $\text{Mn}_4\text{O}_5\text{Sr}$ spectrum is shifted to higher field and centred at $g = 1.980$, similar to the shift observed in W-band spectra from the Bitlt laboratory. The $\text{NH}_3$-modified $\text{Sr}^{2+}$-substituted $S_2$ state signal is centred at $g = 1.979$. The almost identical high-field shift, illustrated by the superimposed $\text{Mn}_4\text{O}_5\text{Sr}$ simulation, indicates that the $G$-tensor shift is dependent only on the presence of $\text{Sr}^{2+}$ but not $\text{NH}_3$. This is in contrast to the result at X-band, which showed that the hyperfine structure is approximately the same for both modifications.

Fig. 3D shows the Q-band Davies $^{55}\text{Mn-ENDOR}$ light-minus-dark spectra, measured at the central magnetic field position of the corresponding EPR spectra (Fig. 3B). The line shape of the $^{55}\text{Mn-ENDOR}$ signal shows only a small field dependence over the 1190 to 1260 mT range (not shown), consistent with its assignment to the tetraneural Mn cluster. In contrast to the X-band CW EPR spectra described above (Fig. 3A), the $S_2$ states of the native, $\text{Sr}^{2+}$-substituted, $\text{NH}_3$-annealed and doubly modified (not shown) OEC give rise to highly similar $^{55}\text{Mn-ENDOR}$ spectra. Five peaks are observed for all three sample types appearing at approximately the same frequency positions and of similar intensities. Small differences in the region of the largest peak ($\approx 115$ MHz) may represent residual $\text{Mn}^{2+}$ contaminations. Importantly, no large difference is seen with regard to the total spectral breadth of the signal ($\approx 55$ to $\approx 195$ MHz). For the $\text{NH}_3$-modified $S_2$ state, the results are nominally consistent with the earlier X-band ENDOR data of Peloquin et al.
The Q-band ENDOR spectra presented here do slightly differ from those presented in ref. 33, 34, 44 and 115 with regard to line intensities, discussed in detail in the Section S6 (ESI†).

Spectral simulations of the complete EPR and 55Mn-ENDOR datasets using the spin Hamiltonian formalism are also shown in Fig. 3 (red dashed lines); the fitted effective $G$ and hyperfine tensors $A$ of the Mn clusters in the low-spin $S = 2$ state are listed in Table 1. The effective $G$ tensors of all three spin systems are in the same range as inferred from EPR measurements on PSII single crystals at W-band, i.e. $G = [1.997, 1.970, 1.965]$ \(^{116}\) and $G = [1.988, 1.981, 1.965]$.\(^{117}\) As found previously,\(^{30,31,33,34,44}\) the inclusion of four hyperfine tensors of approximately the same magnitude and near-axial symmetry is required to simultaneously fit the X-, Q- and W-band EPR and Q-band 55Mn-ENDOR line widths and shapes. The z component represents the principal component for the fitted $G$ and all four hyperfine tensors. Comparison of the fitted parameters demonstrates that the three samples basically exhibit the same electronic structure. The sets of the four isotropic values $A_{i,iso}$ deviate only by $\leq 4\%$ between the three different systems and the signs and magnitudes of the anisotropies $A_{i,aniso}$ are broadly similar, suggesting that there are no significant differences in the electronic exchange coupling schemes of the MnO$_4$Ca/Sr(–NH$_3$) clusters.
### Table 1  Principal values of the effective $G$ and $^{55}$Mn hyperfine tensors $A_i$ for the simulations of the $S_2$ state spectra of the Mn$_4$O$_5$Ca, Mn$_4$O$_5$Sr and Mn$_4$O$_5$Ca–NH$_3$ clusters in PSII from T. elongatus$^*$

<table>
<thead>
<tr>
<th></th>
<th>$G$</th>
<th>$A_1$/MHz</th>
<th>$A_2$/MHz</th>
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<th>$A_4$/MHz</th>
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<td>-74</td>
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</table>

$^a$ All $G$ and $A$ tensors are collinear. $^b$ The equatorial and axial $G$ and $A_i$ values are defined as $G_{z} = (G_{x} + G_{y})/2$, $G_{y} = G_{x}$, and $A_{z} = (A_{x} + A_{y})/2$, $A_{x} = A_{y}$. $^c$ The isotropic $G$ and $A_i$ ($i = 1–4$) values are the averages of the individual values: $G_{iso} = (G_{x} + G_{y} + G_{z})/3$ and $A_{iso} = (A_{x} + A_{y} + A_{z})/3$. $^d$ The anisotropy in the $G$ and $A_i$ values is expressed as the difference $A_{i, aniso} = A_{i, z} - A_{i, x}$.  

#### 3.3 Calculated magnetic properties for the native and modified $S_2$ states of the OEC

The electronic structure of the coupled OEC spin system is defined by the set of six pairwise Mn–Mn exchange interaction terms $J_{ij}$, which can be parameterized using BS-DFT. For all four computational models describing the set of native and chemically perturbed $S_2$ state clusters, the calculations reveal that the $z\beta/z\beta$ spin configuration (Fig. 4A and B) is the lowest in energy. Sets of $J_{ij}$ coupling constants are given in Table S1 of the ESI.$^†$

For all four $S_2$ state OEC forms, the $J$-coupling topology consists of three main coupling pathways (Table S1, ESI.$^†$ and Fig. 4B): (i) an antiferromagnetic coupling pathway between Mn$_{D1}$ and Mn$_{C2}$ ($J_{CD}$); (ii) a ferromagnetic coupling pathway between Mn$_{C2}$ and Mn$_{B3}$ ($J_{BC}$); (iii) and an antiferromagnetic coupling pathway between Mn$_{B3}$ and Mn$_{A4}$ ($J_{AB}$). The ferromagnetic exchange pathway $J_{BC} = 19–28$ cm$^{-1}$ is the largest in absolute magnitude, while the antiferromagnetic pathways $J_{CD} = -16$ to $-18$ cm$^{-1}$ and $J_{AB} = -12$ to $-16$ cm$^{-1}$ are slightly weaker. The remaining exchange coupling constants $J_{BC}$, $J_{AB}$ and $J_{CD}$ are small, as can be expected from geometric considerations (see Table S1, ESI.$^†$). $J_{BC}$ and $J_{CD}$ represent the two largest exchange interactions within the cuboidal trimer unit (Mn$_{D1}$Mn$_{B3}$Mn$_{O3}$) of the cluster, whereas $J_{AB}$ can be considered to a good approximation as being representative of an effective exchange interaction between this cuboidal unit and the outer Mn$_{A4}$, as shown in Fig. 4C.

Systematic differences are observed for the exchange pathways upon the two chemical perturbations, replacement of Ca$^{2+}$ by Sr$^{2+}$ and NH$_3$ exchange at W1 (Table S1, ESI.$^†$). When comparing the structure pairs that differ in terms of the presence of Ca$^{2+}$ or Sr$^{2+}$, i.e. Mn$_{O3}$Ca vs. Mn$_{O3}$Sr and Mn$_{O3}$Ca–NH$_3$ vs. Mn$_{O3}$Sr–NH$_3$, it is seen that only the major coupling pathways $J_{CD}$ and $J_{BC}$ are modified, decreasing by 2 cm$^{-1}$ and 5 cm$^{-1}$, respectively. $J_{AB}$ remains unchanged. By contrast, for the corresponding structure pairs where NH$_3$ is exchanged for W1, the $J_{CD}$ pathway is unchanged, while $J_{BC}$ and $J_{AB}$ increase by 4 cm$^{-1}$. It is noted that the perturbation of the O5 position upon NH$_3$ substitution, as shown in Fig. 2, results also in an enhancement of $J_{BD}$ by 3 cm$^{-1}$. In both cases, the changes in the magnetic interactions can be understood within the geometric changes discussed in Section 3.1 (see Fig. 2): Sr$^{2+}$ substitution mostly affects the structure of the cuboidal unit, thus perturbing principally the exchange pathways within the Mn-trimer unit, whereas NH$_3$ binding perturbs mostly the connectivity between the trimeric moiety and the outer Mn$_{A4}$ (Fig. 4C).
The effective hyperfine couplings of the individual metal ion nuclei scaled by the contribution of the electronic spin of each metal ion to the effective spin state: \( A_i = m_i \rho_i \). The set of scaling factors \( \rho_i \), termed spin projection coefficients, are primarily determined by the set of pairwise exchange couplings as detailed in ref. 18, 30, 31, 33, 34, 36, 37 and 63. However, additional terms must be included to correctly estimate such spin projections for the OEC, specifically the relevant on-site fine structure parameters \( d_i \) for the individual Mn ions, yielding what are more accurately described as spin projection tensors. As the coordination geometries of the three Mn IV ions of the S2 state are all octahedral, their local electronic structure should be of approximate spherical symmetry, their orbitals of \( t_{2g} \) origin \( (d_{xy}, d_{xz}, \text{and } d_{yz}) \) being half-filled (local high-spin \( d^1 \) configuration). As such, the Mn IV ions are expected to only display small fine structure parameters \( d_i \) \((-0.3 \text{ cm}^{-1})^{121}\) and hence do not need to be explicitly considered. Thus, the set of parameters which define the spin projection tensors in the S2 state are the six pairwise interaction terms and the fine structure parameter of the Mn III ion, \( d_{D1} \).

Using these spin projection tensors, the fitted projected Mn hyperfine tensors were scaled back to on-site hyperfine tensors to allow comparison to the BS-DFT values discussed above (Table 2). The only plausible assignment for all three forms of the OEC is that \( A_1, A_2, A_3 \), and \( A_4 \) correspond to \( a_{D1}, a_{A4}, a_{C2} \), and \( a_{B3} \), respectively. In our previous work,\(^{33}\) using BS-DFT structural models predating the latest crystal structure,\(^9\) values of \(-1.2 \text{ to } -1.3 \text{ cm}^{-1} \) were estimated for a supposedly axially symmetric \( d_{D1} \) in the native and \( \text{Sr}^{2+} \)-substituted S2 states. Using the same approach, \( d_{D1} \) was re-estimated. It was possible to obtain on-site hyperfine anisotropies in the ranges characteristic for Mn III and Mn IV ions employing a single value of \(-1.43 \text{ cm}^{-1} \) for the three OEC systems, well within the range typically seen for Mn IV model complexes. As discussed above with regard to the hyperfine tensor anisotropy of Mn D1, a negative \( d \) value requires an effective local \( \text{B}_1 \) state for the Mn D1 ion and is thus consistent with the square-pyramidal 5-coordinate ligand geometry of Mn D1, as present in all computational models. The experimental on-site hyperfine tensor values (Table 2) generally agree well with the BS-DFT estimates and Mn III and Mn IV model compounds, with smaller isotropic values \( a_{D1} \), for the Mn III ion than for the Mn IV ions. In the native and \( \text{Sr}^{2+} \)-substituted models, Mn D1 displays a smaller \( a_{A4,iso} \) than computed and in the \( \text{NH}_3 \)-modified system, where it exhibits the largest coupling of the Mn IV ions. Compared to the calculations, the anisotropic components for the Mn IV ions show a larger variance within \( |a_{D1,iso}| \leq 30 \text{ MHz} \). For Mn D1, \( a_{D1,iso} < -40 \text{ MHz} \) is less negative than calculated. Overall, the experimental results confirm that the computed spin coupling schemes serve as a valid description of the native and modified S2 states.

### 3.4 The Mn D1–His332-imino-N interaction

Three-pulse ESEEM measurements were performed to characterize the imino-N signal of His332 associated with the OEC variants in the S2 state. Fig. S4 and SS (ESIT), respectively, depict \( \tau \) and magnetic-field-dependent \( (g \approx 2.10-1.90) \) light-minus-dark- subtracted spectra and simulations of the native MnO4Ca (A, B), \( \text{Sr}^{2+} \)-substituted MnO4Sr (C, D) and \( \text{NH}_3 \)-modified MnO4Ca-NH3 (E, F) S2 state samples. As noted in Pérez Navarro et al.,\(^{44}\) the 14N nitrogen signal observed for the native S2 state

<table>
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<th>Mn ion</th>
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<th>Spin projections</th>
<th>BS-DFT</th>
<th>Experiment</th>
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<tbody>
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<td></td>
</tr>
<tr>
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<td>-247 – 32 – 204 31</td>
<td></td>
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<tr>
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</tr>
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<td></td>
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</tr>
<tr>
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<td>127 – 142 176 52</td>
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</tbody>
</table>

\( \rho_{iso} \) and \( a_{D1,iso} \) values are the averages of the individual tensor components \( \rho_{iso} = (2\rho_{z} + \rho_{x})/3 \) and \( a_{D1,iso} = (\rho_{x} + \rho_{y} + \rho_{z})/3 \). The anisotropy of the \( \rho_{i} \) and \( a_{D1} \) tensors are expressed as the differences \( \rho_{aniso} = \rho_{z} - \rho_{x} \), and \( a_{aniso} = \rho_{x} - \rho_{y} \); i.e. between the perpendicular and parallel tensor components. The intrinsic fine structure values of Mn IV ions were assumed to be \( d_{A4} = d_{C2} = 0 \text{ cm}^{-1} \). For the Mn III ion, a value of \( d_{D1} = -1.43 \text{ cm}^{-1} \) was fitted, with \( \epsilon_{D1}/d_{D1} = 0 \).
from *T. elongatus* is very similar to that measured in PSII from both higher plants (spinach)\(^ {122}\) and the mesophilic cyanobacteria *Synechocystis* sp. PCC 6803,\(^ {123}\) assigned to the imino-N of His332 via mutagenesis.\(^ {123,124}\) The signals are essentially the same in the native, Sr\(^ {2+}\)-substituted and NH\(_3\)-modified OEC clusters with regard to both their \(\tau\) and magnetic-field dependence. The His332 imino-\(^ {14}\)N signal at Q-band nearly fulfils the cancellation condition, where \(A_{iso}\) is twice the \(^ {14}\)N nuclear Larmor frequency \((\nu_{L} = 3.75 \text{ MHz at 1.22 T})\). The spectra are characterized by three features: the lines centred at frequencies below 2.5 MHz \((\nu_{a} = \nu_{L} - |A_{iso}|/2)\), single-quantum transitions around 7.5 MHz \((\nu_{b} = \nu_{L} + |A_{iso}|/2)\) and smaller double-quantum resonances around 15 MHz \((\nu_{2b} = 2\nu_{L} + |A_{iso}|)\). The line structuring is defined both by the \(^ {14}\)N hyperfine anisotropy and the NQI.

HYSCORE spectroscopy (a two-dimensional ESEEM technique) was performed on the three \(S_{2}\) state OECs at different magnetic-field positions \((g \approx 2.07−1.93)\) of the corresponding Q-band EPR envelopes to further constrain the \(^ {14}\)N hyperfine and quadrupolar interaction matrices. Panels A, C and E in Fig. 5 show the Fourier-transformed spectra and simulations at the centre field position; low- and high-field spectra and simulations are presented in Fig. S6 and S7 in the ESI.† As seen for the three-pulse ESEEM spectra, their appearance is highly similar for all three variants of the OEC in the \(S_{2}\) state. In two dimensions, the three features that comprise the Q-band ESEEM spectra appear as cross peaks at corresponding frequencies both in the \((-,+)^{1}\) and the \((+,+)\) quadrants. As the \(^ {14}\)N hyperfine coupling matches the cancellation condition, the cross peaks are shifted away from the diagonal, instead appearing near the

**Fig. 5** \((-,+)^{1}\) and \((+,+)\) quadrants of the Fourier-transformed Q-band HYSCORE experimental spectra (A, C, E) and spin Hamiltonian-based simulations (B, D, F) of the \(S_{2}\) state \(\text{Mn}_{4}\text{O}_{5}\text{Ca}\) (A, B), \(S_{2}\) state \(\text{Mn}_{4}\text{O}_{5}\text{Sr}\) (C, D) and annealed \(S_{2}\) state \(\text{Mn}_{4}\text{O}_{5}\text{Ca-NH}_{3}\) (E, F) clusters in PSII samples isolated from *T. elongatus* at central magnetic field. SQ and DQ point out the regions of single- and double-quantum transitions, respectively. The optimized parameter sets for the simulations, as described in Section 2.3 and Sections S3, S4 and S8.4 (ESI†), are listed in Table 3 and, in detail, in Table S2 (ESI†). Experimental parameters: microwave frequencies: 34.0370 GHz (Ca), 34.0433 GHz (Sr), 34.0151 GHz (NH\(_{3}\)); magnetic fields: 1220 mT (Ca), 1222 mT (NH\(_{3}\), Sr); shot repetition time: 1 ms; microwave pulse length \((\pi/2)\): 12 ns; \(\tau\): 260 ns; \(\Delta T\): 100 ns; temperature: 5.2 K.
frequency axes. Overall, virtually no orientation dependence is seen comparing the spectra at the three different magnetic fields (Section S8.2, ESI†), consistent with the electron–nuclear interaction being dominated by the isotropic component of the hyperfine coupling as compared to the anisotropic part and the traceless NQI, as in ref. 122, 123 and 125. Thus, the orientation of the His332 imino-14N hyperfine tensor relative to the G tensor cannot be determined from this dataset.

Fitted spin Hamiltonian parameters derived from the simultaneous simulation of both the ESEEM and HYSCORE datasets are given in Table 3 together with BS-DFT estimates. To directly compare DFT values with experiment, the calculated site hyperfine tensor for the His332 was multiplied by the axial MnO21 spin projection tensor described in Section 3.3. All DFT models yield virtually the same hyperfine and quadrupole values. The calculated $A_{\text{iso}}$ underestimates experimental results by <20%, but the dipolar component $A_{\text{dip}}$ and the rhombicity $A_{\text{o}}$ nominally agree with experiment. It is noted that the on-site 14N hyperfine tensor $a$ is expected to be axial with its unique component $a_1$ aligned along the MnO–N bond, as seen in our calculations (Table S2, ESI†). As such, the axial 14N hyperfine component $a_1$ is essentially rotated 90° relative to that of the MnO3 spin projection tensor, which is expected to coincide with the Jahn–Teller axis of MnO13. This 90° rotation explains why the 14N hyperfine tensor $A$ is rhombic in the projected (experimental) reference frame. For a more detailed description of the simulations, see Section S8.4 (ESI†). Importantly, the near-invariance of the imino-$N$ spin Hamiltonian parameters for the three $S_2$ state forms requires the His332 ligation, the electronic structure of the MnO311 ion and by extension the whole tetranuclear Mn cluster, to not be significantly perturbed by Ca+2/Sr+2 and NH3/W1 exchange, in line with the EPR/55Mn ENDOR results described in Section 3.2.

While the orientation of the hyperfine tensor relative to the $G$ tensor cannot be determined using ESEEM/HYSCORE at Q-band frequencies, it can be measured at W-band, e.g. using EDNMR. In our earlier study,14 it was found that the hyperfine tensor is oriented such that its principal, i.e. the smallest component $A_1$ is aligned such that it is mid-way between $G_x$ and $G_z$. Importantly though, it is noted that the set of spin Hamiltonian parameters deduced from Q-band ESEEM/HYSCORE (Table 3) does not reproduce the W-band data sets (Section S8.3, ESI†). This is not due to the inclusion/exclusion of the NQI term, which, for the W-band EDNMR data, mainly contributes to the spectral line width. To reproduce the field dependence of the 14N- and 15N-EDNMR signals (Fig. S8 and S9, ESI†), the values determined from Q-band ESEEM/HYSCORE needed to be scaled: $A_{\text{iso}}$ was decreased by 10%, whereas $A_{\text{dip}}$ was increased by a factor of two (Table S2, ESI†). The same results were observed for all three $S_2$ state forms, which basically exhibit the same 14N-EDNMR spectra. A possible reason for this difference comes from the observation that the ground spin state, an effective spin $S_T$ = 1/2 state, is not very well separated energetically from higher spin states in the regime of the W-band excitation energy ($\approx 3$ cm$^{-1}$), consistent with DFT estimates for the ground-to-first excited state energy splitting $\Delta E$ (Section S7, ESI†). Excited-state mixing due to a small $\Delta E$ has the consequence of altering spin Hamiltonian observables such as effective 55Mn and 14N hyperfine tensors. Alternatively, the rhombicity of the effective G tensor as inferred from the EPR/55Mn-ENDOR simulations may be artificial, a consequence of using collinear $G$ and 55Mn hyperfine tensors. This latter suggestion would also explain why the $G$ tensors inferred from W-band measurements on PSII single crystals16,117 differ from those inferred from our multifrequency measurements on frozen solution PSII samples.

The lack of agreement between the two 14N datasets brings into question whether the W-band 14N/15N-EDNMR signals can be used to assign the exchangeable μ-oxo bridge 17O signal based on the relative orientations of the 14N and 17O hyperfine tensors, as suggested by Rapatskiy et al.14 Thus, further experimental results, particularly from single crystals of PSII, are needed to test this proposal (see Section 3.6).

Table 3  Fitted and calculated effective/projected 14N hyperfine and NQI tensors in MHz for the electron–nuclear couplings of the His332 imino-$N$ and of NH3 with the various cluster forms studied in the $S_2$ state in PSII from T. elongatus

| $S_2$ state   | 14N Method | $A_{\text{iso}}^a$ | $A_{\text{dip}}$ | $A_{\text{o}}$ | $|\epsilon|^2Qg/h$ | $\eta^c$ |
|---------------|-----------|-------------------|-----------------|-------------|----------------|--------|
| Native        | Exp.      | 7.1               | 0.75            | 0.81        | 1.97           | 0.75   |
|               | DFT       | 5.8               | 0.59            | 0.74        | 1.65           | 0.91   |
| Sr2+-substituted | Exp.      | 7.3               | 0.69            | 0.83        | 1.98           | 0.79   |
|               | DFT       | 5.8               | 0.57            | 0.61        | 1.65           | 0.91   |
| NH3-modified  | Exp.      | 7.2               | 0.75            | 0.89        | 1.96           | 0.80   |
|               | DFT       | 6.1               | 0.71            | 0.99        | 1.68           | 0.88   |
| NH3-modified, Sr2+-substituted | Exp.      | 2.36              | 0.33            | 0.22        | 1.52           | 0.47   |
|               | DFT       | 2.68              | –0.65           | 0.02        | 0.94           | 0.87   |

$A_{\text{iso}}$ is defined as the average of the principal components of the hyperfine tensor: $A_{\text{iso}} = (A_1 + A_2 + A_3)/3$. $A_{\text{dip}}$ is defined in terms of $T_1$, $T_2$, and $T_3$ as $A_{\text{dip}} = (T_1 + T_2)/2 = –T_3/2$. The rhombicity is defined by $A_{\text{o}}$ or $\eta = (T_1 – T_2)/T_3$, respectively. $T_1$, $T_2$, and $T_3$ represent the three principal components of the hyperfine tensors minus $A_{\text{iso}}$ and of the NQI tensors and are labelled such that $|T_1| \leq |T_2| \leq |T_3|$. The Euler rotation angles $[\alpha, \beta, \gamma]$ of the NQI relative to the $A$ tensors are $[20, 12, 0]^c$, $[18, 9, 0]^c$ and $[16, 16, 0]^c$ for the MnO$_2$Ca, MnO$_2$Sr and MnO$_2$Ca–NH$_3$ clusters, respectively.
3.5 NH₃ binding to the Ca²⁺- and the Sr²⁺-containing OEC

In the NH₃-modified S₂ state, a second nitrogen nucleus is bound to the Mn cluster as a terminal ligand, as described in Pérez Navarro et al. Its binding can be observed using X-band (three-pulse) ESEEM, as shown in Fig. S11 (ESI†) for ¹⁴NH₃/¹⁵NH₃-bound, Ca²⁺- and Sr²⁺-containing PSII. The ¹⁴NH₃ resonances comprise three characteristic single-quantum lines at 0.5, 0.95 and 1.45 MHz split by the NQI and smaller double-quantum transitions centred at 4.9 MHz, highly similar to the higher plant data. Due to the lack of the NQI, the ¹⁵NH₃ signal is clearly less complicated, consisting only of one single-quantum hyperfine peak centred at 0.3 MHz. As seen for the His332 imino-¹⁴N signal at Q-band, the NH₃ interaction at X-band fulfils the cancellation condition, leading to a narrow ν₀ line while the ν₁ line is broadened beyond detection. Most importantly, the spectra of the ¹⁴NH₃-modified Ca²⁺- and Sr²⁺-containing ¹⁴N-PSII samples are essentially identical. Thus, NH₃ binding to the Sr²⁺-substituted S₂ state cluster is the same as in the native S₂ state.

In our first report on NH₃ binding to the OEC, only the ¹⁴NH₃ interaction was considered. Here, we simultaneously fit the spectra of both the ¹⁴NH₃-modified ¹⁵N-PSII and the ¹⁵NH₃-modified ¹⁴N-PSII in the S₂ state (Table 3, Fig. S11 and Table S3, ESI†). This resulted in an optimized hyperfine tensor A = [2.76 2.62 1.69] MHz for ¹⁴NH₃ (and for ¹⁵N scaled by the ratio of the nuclear g values of ¹⁵N and ¹⁴N) and ¹⁴N NQI parameters e²Qgh = 1.52 MHz and η = 0.47. Highly similar hyperfine and NQI values reproduce the NH₃ signal observed in the annealed S₂ state of Sr²⁺-containing ¹⁴N-PSII. The isotropic ¹⁴N hyperfine coupling Aiso = 2.36 MHz and Adip = 0.33 MHz, e²Qgh/η and the asymmetry parameter η are the same as reported before for PSII from T. elongatus and similar to the values from the analysis of higher plant X-band ESEEM spectra† (Table 3).

An axial projected ¹⁴NH₃ hyperfine tensor is obtained from BS-DFT calculations, as seen in the experiment. This is because (i) the on-site ¹⁴NH₃ hyperfine tensor is axial, and (ii) its axial and equatorial components are essentially coincident with those of the MnA₄ spin projection tensor (Table S3, ESI†), unlike the case for the His332 imino-¹⁴N a tensor (see Section 3.4). The BS-DFT calculations also reproduce the comparably large and rhombic NQI parameters (Table 3), although the sign of the hyperfine anisotropy is inverted compared to experiment. For more details, see Section S9 in the ESI.†

3.6 Interactions with exchangeable ¹⁷O species

As we have recently shown, EDNMR spectroscopy at W-band, due to its comparatively high sensitivity, is the preferred method to measure the interactions of exchangeable ¹⁷O nuclei with fast-relaxing electronic species such as the S₁ state of the OEC. Fig. 6 shows these spectra and simulations (see Section 2.3 and Sections S3 and S4, ESI† for details) of the single-quantum region for the native, the Sr²⁺-substituted, the NH₃-annealed and the Sr²⁺- and NH₃-modified S₂ state variants after H₂O/¹⁷O buffer exchange in the S₁ state (see Fig. S12 (ESI†) for the double-quantum region). The spectrum of the native system exhibits the single- and double-quantum resonances of the imino-¹⁴N of His332 (blue) and of three different classes of ¹⁷O species, i.e. (i) a strongly coupled, bridging species (green), (ii) an intermediate coupled terminal O-ligand (orange), and (iii) a weakly coupled terminal class (cyan). These were assigned to the μ-oxo bridge O5, the hydroxide ion W2²⁺ and the H₂O matrix (comprising ligand W1 of MnA₄ and two H₂O ligands at the Ca²⁺ ion), respectively. NH₃ binding causes a narrowing of the ¹⁷O single- and double-quantum envelopes, reproduced by a decrease of the hyperfinecouplings of O5 and W2 and concomitant reduction of the matrix line intensity, which was interpreted by NH₃ binding to MnA₄ in exchange for W1. Comparing these two spectral forms to those of the corresponding Sr²⁺-substituted W₁- and NH₃-containing clusters (Fig. S13, ESI†), we see a systematic narrowing of the single-quantum envelope by ≈ 0.5 MHz and a corresponding narrowing of the double-quantum envelope. This can be reproduced by spectral simulations in which the hyperfine couplings of the μ-oxo bridge are reduced accordingly (W1: 9.2 MHz vs. 9.7 MHz, NH₃: 6.5 vs. 7.0 MHz), while the other ¹⁷O interactions remain unaltered (for a complete set of hyperfine parameters, see Table S4, ESI†). Although weaker than the NH₃ effect, the
narrowing was found to be reproducible in all Sr\(^{2+}\)-substituted PSII samples. It clearly shows that Ca\(^{2+}\)/Sr\(^{2+}\) exchange perturbs the \(\mu\)-oxo bridge, in addition to a simultaneous modification by NH\(_3\) binding.

The inset in Fig. 6 depicts a section of the X-Band CW EPR spectra of the Sr\(^{2+}\)-substituted \(S_2\) state, which exhibits an intrinsically smaller average line width (\(\approx 3.6\) mT peak-to-peak) than the native form (\(\approx 4\) mT), in the presence and absence of \(^{17}\)O (see Fig. S14 (ESI\(^*\)) for the entire spectra). No EPR line broadening is observed upon \(^{17}\)O exchange. This demonstrates that the largest \(^{17}\)O coupling represents only one exchangeable oxo bridge. In the case of two hyperfine interactions of \(\approx 10\) MHz, the effective line broadening due to the combined \(^{17}\)O couplings would be larger than 120 MHz or 4.3 mT, exceeding the actual line width.

### 4 Discussion

#### 4.1 A common electronic structure of the \(S_2\) state variants

Our DFT results show that the Sr\(^{2+}\)-substituted, the NH\(_3\)-annealed and the Sr\(^{2+}\)- and NH\(_3\)-modified low-spin \(S_2\) states basically represent the same structure on both a geometric and electronic level. This result is not immediately obvious from their X-band EPR signals. Indeed historically, the Sr\(^{2+}\)-substituted and NH\(_3\)-modified forms were explained in terms of a change of the valence state distribution within the Mn tetramer and thus of the coordination environment of the Mn\(^{III}\) ion.\(^{126}\) The comprehensive approach pursued in this study conclusively rules out such a mechanism for electronic structure perturbation. Instead, as proposed by our group,\(^{33,34,63}\) multiline heterogeneity reflects rather subtle changes of the Mn-tetramer structure. The similarity of the perturbed multiline forms suggest a common mechanism for electronic perturbation, which probably also explains \(S_1\) state heterogeneity. This is discussed below, with reference to solvent access, substrate binding and exchange.

#### 4.1.1 The mechanism of structural perturbation

In Su et al.,\(^{34}\) a qualitative model for multiline heterogeneity was proposed. In this model, the electronic structure of the \(S_2\) state was considered in terms of a simplified model of two spin fragments: (i) a cuboidal trimer unit, made up of Mn\(_{D1}\), Mn\(_{C2}\) and Mn\(_{B3}\), and (ii) a ‘monomeric’ Mn unit, consisting of the outer Mn\(_{AA}\). Therein, variation of the electronic structure in \(S_2\) was attributed to changes in the connectivity of the outer Mn\(_{AA}\) to the cuboid, changing the properties of the electronic ground state by altering the mixing-in of excited spin state character. The physical rationale for this observation was that the outer Mn\(_{AA}\) represents the solvent accessible end of the cluster. It has two water-derived ligands and solvent channels that begin or terminate at this site.\(^{9,127}\) As such, it is this site and its connection to the rest of the cluster that is most likely to vary amongst different sample conditions and possibly different PSII species. In terms of this ‘monomer–trimer’ model with regard to the two modifications discussed here, Ca\(^{2+}\)/Sr\(^{2+}\) forms part of the linkage between the cuboidal and outer fragments, as mediated by the \(\mu\)-oxo bridge O5, whereas NH\(_3\) binds to the outer Mn\(_{AA}\) also perturbing O5, the bridge \(trans\) to its binding position W1.\(^{44}\)

The magnetic observable that is altered upon Ca\(^{2+}\)/Sr\(^{2+}\) replacement and/or \(W1/NH_3\) exchange, leading to the perturbed multiline forms is the \(^{55}\)Mn hyperfine anisotropy. Small perturbations of the four hyperfine tensors result in a change in the hyperfine peak superposition, altering the apparent structure of the X-band EPR signal (Fig. 3A). Importantly these changes are subtle, as demonstrated by the invariance of the \(^{55}\)Mn-ENDOR spectra (Fig. 3D). The \(^{55}\)Mn hyperfine anisotropy is not a site property, but instead an indirect measure of the fine structure splitting of the Mn\(^{III}\) ion\(^{10,13,34,63}\) or, in the ‘monomer–trimer’ model, the zero-field splitting of the whole trimer unit. Within this model, its contribution is modulated by the electronic connectivity between the two fragments, predominantly the exchange pathway \(J_{AB}\) the coupling that mostly defines the energy splitting \(\Delta E\) between the ground state and the first excited state (Fig. 4C). Our BS-DFT results support this basic mechanism for electronic structure perturbation and, for the first time, describe the changes on the molecular level that impart this variation, and which differ for the two modifications. Upon replacement of Ca\(^{2+}\) by Sr\(^{2+}\), the slight distortion of the cuboidal moiety leads to a perturbation of the intra-cuboidal exchange network and possibly the Mn\(_{D1}\) site fine structure splitting. It is noted that this, besides changing the \(^{55}\)Mn hyperfine anisotropy, also manifests itself in terms of the \(G\) tensor, also contributing to the altered multiline appearance and the \(g\) shift of the W-band EPR signal. Exchange of W1 by NH\(_3\) affects the connectivity of the outer Mn\(_{AA}\) to the cuboidal unit, as modulated by the \(\mu\)-oxo bridge O5, perturbing the \(J_{AB}\) exchange pathway, thus changing the \(^{55}\)Mn hyperfine anisotropy. In the case of the \(S_2\) state variant that contains both these modifications, the Mn\(_{AA}\) is perturbed by NH\(_3\) binding, which perturbs the Mn\(_{AA}\) site. This is part of the trimer fragment in the \(S = 1/2\) configuration, that should form the dominant contribution to the anisotropy of the \(G\) tensor in all four systems.

#### 4.1.2 The Mn\(_{D1}\)-His332-imino-N and Mn\(_{AA}\)-NH\(_3\) interactions as local probes for the electronic structure

##### 4.1.2a The Mn\(_{D1}\)-His332-imino-N interaction

As described by Stich et al.,\(^{123}\) the magnitude of the Mn\(_{D1}\)-His332 imino-N hyperfine interaction, as compared against mixed-valence Mn\(^{III}\)Mn\(^{IV}\) model compounds and protein cofactors with imidazole ligands to Mn\(^{III}\) (\(A_{iso} \leq 13\) MHz) and Mn\(^{IV}\) ions (\(A_{iso} = 1.5–3.3\) MHz),\(^{120,134}\) favouring assigning Mn\(_{D1}\) as the only Mn\(^{III}\) ion of the \(S_2\) state, consistent with the EPR/\(^{55}\)Mn-ENDOR/DFT results already reported in the literature and detailed above (Sections 3.1–3.3). The large hyperfine couplings seen for ligands coordinating to Mn\(^{III}\) in \(S_2\) (and model systems) comes from the fact that the Mn\(^{III}\) ion carries the largest spin projection coefficient, \(i.e.,\) in Mn dimers \(\rho_{iso}(\text{Mn}^{III}) = 2\) and \(\rho_{iso}(\text{Mn}^{IV}) = –1\). Interestingly, the hyperfine and quadrupole couplings of imidazole ligands of Mn\(^{III}\) ions differ depending on whether they represent axial (\(A_{iso} = 9–13\) MHz, \(e^2Qq/h = 2.1–3.0\) MHz)\(^{128,132}\) or equatorial ligands (\(A_{iso} = 1.5–6.6\) MHz, \(e^2Qq/h = 1.5–2.5\) MHz).\(^{131,134}\) The values seen for the His332...
Both modifications investigated, Ca$^{2+}$/Sr$^{2+}$ substitution and NH$_3$/W1 replacement, perturb the $^{17}$O-EDNMR signals of the exchangeable oxygen species of the OEC, specifically the exchangeable $\mu$-oxo bridge. It is this bridge that likely represents one of the substrate water sites of the Mn tetramer. As the electronic structure of the OEC is essentially invariant for all four OEC forms, the change in hyperfine coupling for this $\mu$-oxo bridge must represent a site modification, near or at the oxygen nucleus. NH$_3$ binding primarily affects the connectivity of the outer Mn$_{A4}$ to the cuboidal trimer, whereas Sr$^{2+}$ substitution instead perturbs the exchange network within the cluster. Thus, it can be surmised that the exchangeable $\mu$-oxo bridge must both coordinate to the outer Mn$_{A4}$ and be associated with the Ca$^{2+}$/Sr$^{2+}$ ion itself as a structural element of the cuboidal trimer. Only the bridge O5 fulfills both these criteria. As a ligand to the Ca$^{2+}$/Sr$^{2+}$ ion, O5 is affected by the exchange of these ions of the same charge but different sizes. Similarly, as argued in Pérez Navarro et al.,$^{44}$ NH$_3$/W1 exchange perturbs O5 by binding trans to this bridge position, distorting the Mn$_{A4}$–O5 bond length.

It is noted that these results exclude the possibility that NH$_3$ displaces the exchangeable $\mu$-oxo bridge as a bridging –NH$_2$– species, an alternative rationale for the narrowing of the $^{17}$O signal envelope in line with earlier suggestions.$^{70}$ Ca$^{2+}$/Sr$^{2+}$ and W1/NH$_3$ exchange are additive in terms of their effect on the width of the $^{17}$O-EDNMR envelope, modelled here as defined by the $\mu$-oxo bridge hyperfine coupling $A_{iso}$. This result mirrors the structural modifications observed for the doubly modified Mn$_4$O$_5$Sr–NH$_3$ OEC model; i.e., the model contains additive structural modifications reflecting both singularly modified Mn$_4$O$_5$Sr and Mn$_4$O$_5$Ca–NH$_3$ structures. If instead the NH$_3$ did indeed replace the bridge, the width of the $^{17}$O-EDNMR envelope would be now defined by the W2 hyperfine coupling, and as such should be invariant to Ca$^{2+}$/Sr$^{2+}$ substitution. It is also noted that NH$_3$ replacement of the exchangeable bridge O5 cannot quantitatively explain the virtually unaltered $^{17}$NH$_3$ signal upon exchange of the O5-binding Ca$^{2+}$, and the $^{17}$O hyperfine changes. Assuming an unaltered spin density on the bridge position, as follows from the similar spin projection factors for the four Mn ions, the measured $^{14}$N hyperfine coupling for the bound ammonia at this position is far too small. On the other hand, the $^{17}$O coupling of 6.5–7 MHz seen for the NH$_3$/W1-exchanged system is in the range of those observed in Mn model complexes with a N-ligand trans to the oxo bridge.$^{14}$

5 Conclusions

Time-dependent mass spectrometry experiments indicate that the early binding substrate [W$_S$] is associated with all intermediate states of the OEC.$^{66,136}$ Furthermore, the relatively slow exchange and the S-state dependence of this bound substrate with bulk water suggests that it represents a ligand of (a) Mn ion(s). As Ca$^{2+}$/Sr$^{2+}$ substitution also perturbs its exchange rate, W$_S$ is also supposed to coordinate to the Ca$^{2+}$ ion.$^{55,66}$ Of the exchangeable oxygen species identified here by $^{17}$O-EDNMR, only O5 is a ligand to both Mn and Ca$^{2+}$. Similarly, only the O5 spectral signature is perturbed by Ca$^{2+}$/Sr$^{2+}$ exchange. Thus, O5 is the most likely candidate for W$_S$. This assignment limits the
possible reaction pathways for photosynthetic water splitting, and lays a foundation for studies of higher oxidized S states, which will serve to identify the second, fast exchanging substrate and eventually elucidate the mechanism of O–O bond formation. Currently two pathways are envisaged: O–O bond formation could proceed as a coupling between O5 and either (i) Mn4+-bound W2 or Ca2+-bound W3, or (ii) a further oxygen not present yet in the S2 state.

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Notes and references