The Basic Properties of the Electronic Structure of the Oxygen-evolving Complex of Photosystem II Are Not Perturbed by Ca$^{2+}$ Removal*

Thomas Lohmiller¹, Nicholas Cox⁰, Ji-Hu Su¹, Johannes Messinger², and Wolfgang Lubitz²,³

From the Max-Planck-Institut für Bioanorganische Chemie, Stiftstrasse 34-36, 45470 Mülheim an der Ruhr, Germany and the Department of Chemistry, Chemical Biological Centre (KBC), Umeå University, 90187 Umeå, Sweden

Background: EPR/$^{55}$Mn ENDOR spectroscopy of the oxygen-evolving complex (OEC) and Mn$^{2+}$ in Ca$^{2+}$-depleted photosystem II.

Results: Electronic model of the Ca$^{2+}$-depleted OEC; characterization of Mn$^{2+}$ binding.

Conclusion: Ca$^{2+}$ is not critical for maintaining the electronic and spatial structure of the OEC. Its removal exposes a Mn$^{2+}$ binding site supposedly in an extrinsic subunit.

Significance: Mechanistic implications for water oxidation; Mn$^{2+}$ in photoassembly/D1 protein repair.

Ca$^{2+}$ is an integral component of the Mn$_4$O$_5$Ca cluster of the oxygen-evolving complex in photosystem II (PS II). Its removal leads to the loss of the water oxidizing functionality. The S$_2$ state of the Ca$^{2+}$-depleted cluster from spinach is examined by X- and Q-band EPR and $^{55}$Mn electron nuclear double resonance (ENDOR) spectroscopy. Spectral simulations demonstrate that upon Ca$^{2+}$ removal, its electronic structure remains essentially unaltered, i.e., that of a manganese tetramer. No redistribution of the manganese valence states and only minor perturbation of the exchange interactions between the manganese ions were found. Interestingly, the S$_2$ state in spinach PS II is very similar to the native S$_2$ state of Thermosynechococcus elongatus in terms of spin state energies and insensitivity to methanol addition. These results assign the Ca$^{2+}$ a functional as opposed to a structural role in water splitting catalysis, such as (i) being essential for efficient proton-coupled electron transfer between Y$_Z$ and the manganese cluster and/or (ii) providing an initial binding site for substrate water. Additionally, a novel $^{55}$Mn$^{2+}$ signal, detected by Q-band pulse EPR and ENDOR, was observed in Ca$^{2+}$-depleted PS II. Mn$^{2+}$ titration, monitored by $^{55}$Mn ENDOR, revealed a specific Mn$^{2+}$ binding site with a submicromolar $K_D$ Mn$^{2+}$ titration of Mn$^{2+}$-loaded, Ca$^{2+}$-depleted PS II demonstrated that the site is reversibly made accessible to Mn$^{2+}$ by Ca$^{2+}$ depletion and reconstitution. Mn$^{2+}$ is proposed to bind at one of the extrinsic subunits. This process is possibly relevant for the formation of the Mn$_4$O$_5$Ca cluster during photoassembly and/or D1 repair.

The oxygen-evolving complex (OEC) of photosystem II (PS II) catalyzes the light-driven oxidation of water. The OEC contains an inorganic Mn$_4$O$_5$Ca metallocofactor that includes five μ-oxo bridge linkages and is coordinated by a framework of surrounding amino acids (1–6) in a highly defined manner that confers catalytic function. The redox-active tyrosine residue Y$_Z$ (D1-Tyr-161) enables electron transfer from the Mn$_4$O$_5$Ca cluster to P$_{680}$$^+$, the radical cation formed upon photon absorption and charge separation. The Mn$_4$O$_5$Ca cluster undergoes four successive oxidations, cycling through a series of different net valence states, referred to as the S$_i$ states (where $i = 0–4$ denotes the number of oxidizing equivalents stored in the cluster). The transient state S$_4$ spontaneously returns to S$_0$ upon regaining four electrons from the two substrate water molecules, which in the process form molecular oxygen. The release of O$_2$ is followed by the rebinding of at least one H$_2$O molecule (for reviews, see Refs. 7–14).

X-ray crystallographic structures of the PS II protein complex provided an atomic picture of the structure of the OEC (1–6), identifying all amino acids that ligate the Mn$_4$O$_5$Ca cluster. The metallocofactor resembles a distorted chair, consisting of the cuboidal moiety Mn$_3$O$_3$Ca (Mn$_{63}$Mn$_{c2}$Mn$_{D1}$), with the fourth, outer manganese ion (Mn$_{D4}$), connected to the cuboid via an additional μ-oxo bridge (O4) to one of the manganese vertices (Mn$_{D3}$). The reported cluster is likely modified due to photoreduction of the Mn$^{III}$ and Mn$^{IV}$ ions, such that the Mn-Mn and Mn-Ca distances seen in the x-ray structure are all elongated as compared with those derived from extended x-ray absorption fine structure (EXAFS) measurements (15). Allowing for this, the basic topology of the x-ray structure is similar to earlier literature models, including the geometry-optimized.

* This work was supported by Max Planck Gesellschaft and the EU/Energy Network project SOLAR-H2 (FP7 Contract 212908).

This article contains supplemental Equation S1, Table S1, and Figs. S1–S4.

¹ Supported by the Federal Ministry of Education and Research of Germany (BMBF) in the framework of the Bio-H2 project (03SF0355C).

² Supported by the National Natural Science Foundation of China (31070211). Present address: Dept. of Modern Physics, University of Science and Technology of China, Hefei, Anhui 230026, China.

³ Supported by Vetenskapsrådet, Umeå University (Solar Fuels Strong Project Umeå), and the Kempe foundation.

4 To whom correspondence should be addressed. Tel.: 49-208-306-3614; Fax: 49-208-306-3955; E-mail: wolfgang.lubitz@mpi-muel.mpg.de.

5 The abbreviations used are: OEC, oxygen-evolving complex; PS II, photosystem II; EXAFS, extended X-ray absorption fine structure; ENDOR, electron-nuclear double resonance; CW, continuous wave; RF, reaction center; ESE, electron spin echo; RF, radio frequency; HFI, hyperfine interaction; ZFS, zero-field splitting; DFT, density functional theory; mT, militesla; MW, microwaves; μ, arbitrary units.

6 The nomenclature used for the manganese ions combines the lettering/numbering used in polarized EXAFS models (90) and that of Umena et al. (6).
Electronic Structure of the Ca\(^{2+}\)-depleted OEC of Photosystem II

density functional theory (DFT) models of Kusunoki (16), Siegbahn (17), and the recent model of Ames et al. (18), in which the cuboid exhibits an open conformation with Mn\(\text{A}_4\) connected to Mn\(\text{B}_3\) via a di-\(\mu\)-oxo bridge (Fig. 1).

The Ca\(^{2+}\) ion of the Mn\(_{4}\)O\(_{5}\)Ca cluster, which can be removed from and reconstituted into the OEC (19–21), is essential for catalytic function (19–23). The non-catalytic Ca\(^{2+}\)-depleted OEC cannot complete the S state cycle, advancing only to a modified S\(_2\) state, termed S\(_2'\) (24, 25). The reason for this remains unclear. However, four basic explanations exist in the current literature based on the proposed role(s) for the Ca\(^{2+}\) ion during the S state cycle (for reviews, see Refs. 26–28). These include the following: (i) As an integral component of the OEC (6), the Ca\(^{2+}\) ion can be suspected to be of crucial structural importance. However, EXAFS experiments suggest that Ca\(^{2+}\) depletion leads to only a small spatial reorganization of the remnant Mn\(_{4}\)O\(_{5}\) cluster (29). (ii) It facilitates fast one-electron transfer from the OEC to Y\(_{2}\)\(^{+}\) (for reviews, see Refs. 11 and 30). The formation of the S\(_2'\) state requires long visible light illumination, which could lead to a decoupling of the cluster or a rearrangement of the manganese valence states. Thus, Ca\(^{2+}\) depletion could potentially change the redox properties as well as substrate and/or protein interactions of the complex, inhibiting catalytic function.

The Mn\(_{4}\)O\(_{5}\)Ca cluster in the S\(_2\) state exhibits a characteristic multiline EPR signal centered at \(g \approx 2\) (33) that arises from an \(S = 1/2\) ground spin state of the cluster. Under certain conditions (illumination, reactants), additional signals are observed at higher \(g\) values; in spinach, a second broad signal can be detected at \(g = 4.1\) (34, 35), attributed to an \(S = 5/2\) spin state (36). These signals are affected by the presence of small alcohols, foremost methanol (MeOH) (37–41), which enhance the intensity of the multiline signal at the expense of the \(g \approx 4.1\) signal (37) (for a full discussion see Ref. 41). The Mn\(_{4}\)O\(_{5}\) cluster in the S\(_2'\) state also exhibits a multiline signal; however, its hyperfine splitting pattern is perturbed. It contains a larger number of resolved lines as compared with the native S\(_2\) multiline signal, with a smaller average line spacing (5.5–6 versus 8.8 mT). The magnetic interaction between Y\(_{2}\) and the OEC is also perturbed in Ca\(^{2+}\)-depleted PS II as evidenced by changes in the tyrosine split signal of the S\(_2\) Y\(_{2}\) state (24, 25).

A detailed understanding of the electronic structure of the Mn\(_{4}\)O\(_{5}\)Ca cluster in the S\(_2\) state has been developed from pulse EPR data (42–46), in particular \(^{55}\)Mn electron nuclear double resonance (ENDOR). These experiments demonstrated that the four manganese ions contribute about equally to the ground electronic state of the S\(_2\) state; i.e., all four manganese ions carry approximately the same spin density. This requirement allows an assessment of the electronic exchange interactions between the four manganese ions and the development of Mn\(_{4}\) coupling schemes. These necessarily reflect the geometric structure of the OEC and allow the assignment of the individual manganese oxidation states. Our recently proposed model for the S\(_2\) state (18) is described under “Discussion.” This scheme places the only Mn\(^{3+}\) ion inside the cuboidal unit (Mn\(_{4}\)D\(_1\)) (see also Ref. 47) and compares favorably with information from complementary spectroscopic measurements (48–50).

Although it has not been directly observed by EPR spectroscopy, the possibility of another paramagnetic manganese species being able to bind to the Ca\(^{2+}\)-depleted PS II has been suggested in an earlier study by Booth et al. (51). The additional species was suggested to be a Mn\(^{2+}\) ion that can bind specifically to a site in the protein complex that is created or becomes accessible via structural changes in the course of Ca\(^{2+}\) removal. This was based on the observation that, after equimolar amounts of Mn\(^{2+}\) ions had been added to Ca\(^{2+}\)-depleted PS II, no Mn\(^{2+}\) was observed by X-band continuous wave (CW) EPR. Upon titrating Ca\(^{2+}\) ions back into these samples, Mn\(^{2+}\) was released as seen from the appearance of the six-line Mn\(^{2+}\) EPR signal.

In this work, both the spin system of the Mn\(_{4}\)O\(_{5}\) cluster in the S\(_2'\) state of Ca\(^{2+}\)-depleted PS II and the binding of Mn\(^{2+}\) ions to this protein were studied by EPR and ENDOR spectroscopy at X- and Q-band frequencies. The results provide new insight into the role of the Ca\(^{2+}\) ion in the native OEC.
**TABLE 1**

<table>
<thead>
<tr>
<th>Observable</th>
<th>Native</th>
<th>Ca(^{2+})-depleted</th>
<th>Ca(^{2+})-reconstituted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic rates/µmol O(_2)/mg chlorophyll/h(^a)</td>
<td>390 ± 30</td>
<td>27 ± 1</td>
<td>330 ± 30</td>
</tr>
<tr>
<td>Relative enzymatic rates</td>
<td>100%</td>
<td>7 ± 0%</td>
<td>84 ± 8%</td>
</tr>
<tr>
<td>Relative S(_2) state multiline signal intensities(^b)</td>
<td>100%</td>
<td>8 ± 3%</td>
<td>105 ± 12%</td>
</tr>
</tbody>
</table>

\(^a\) Determined as an average of at least 8 single measurements at a minimum of 2 different chlorophyll concentrations from 5 to 25 µg/ml. 

\(^b\) Determined from the peak-to-trough distances of four prominent derivative peaks in the CW EPR spectrum (100).

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—PS II-enriched thylakoid membranes were prepared from spinach based on the procedure of Berthold et al. (52) using detergent treatment by incubation with Triton X-100 for 15 min. All work was performed in the dark or very dim green light, and the PS II was kept at 4 °C before storage in the dark at −80 °C or in liquid N\(_2\). Chlorophyll concentrations were determined by assays using aqueous acetone (80%) extracts (53) with updated extinction coefficients (54) using an ATI Unicam UV-visible spectrometer UV2–300.

**Ca\(^{2+}\)** depletion and reconstitution based on the low pH/citrate treatment method (21) was achieved as described previously (55). The final buffer used was 50 mM MES, 15 mM NaCl, 0.4 M sucrose, 1 mM EDTA, pH 6.5. Ca\(^{2+}\) removal and, as a proof for the integrity of the OEC, Ca\(^{2+}\) rebinding was confirmed both by enzymatic assays and by X-band CW EPR. The O\(_2\) evolution rates of native PS II were ~400 µmol O\(_2\)/mg of chlorophyll/h (see the following section). O\(_2\) evolution rates dropped to 5–10% in Ca\(^{2+}\)-depleted and were reactivated to >80% in Ca\(^{2+}\)-reconstituted samples. Similar percentages of the S\(_2\) multiline signal were observed after white light illumination with a tungsten lamp through an aqueous 5% CuSO\(_4\) IR filter of the respective samples at 200 K for 5 min (Table 1, Fig. 2A). These numbers are consistent with previous literature reports (25, 29, 56).

Advancement of dark-adapted S\(_1\)' state EPR samples to the S\(_2\)' and S\(_2\)'Y\(_Z\)' states (25) was done by illumination at 0 °C for 3 min, with 125 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (10 mM in dimethyl sulfoxide) added to the samples advanced to the S\(_2\)' state, which restricts the acceptor site and, thus, Y\(_Z\) to one turnover.

For Ca\(^{2+}\) and Mn\(^{2+}\) titration experiments, dark-adapted Ca\(^{2+}\)-depleted PS II membranes were rebuffered in EDTA-free buffer by three cycles of dilution, centrifugation at 39,000 × g for 15 min, and resuspension using 50 mM MES, 15 mM NaCl, 5 mM MgCl\(_2\), 0.4 M sucrose, pH 6.5. The final concentration of PS II reaction centers (RCs) in the samples was 28 ± 3 µM based on a chlorophyll concentration of 6.3 ± 0.8 mg ml\(^{-1}\) and assuming 250 chlorophylls/RC (57) after 15 min Triton X-100 treatment. The samples were incubated with known amounts of Mn\(^{2+}\) ranging from 0 to 4 eq per RC for 2 h. For the Ca\(^{2+}\) titration, samples containing 0.8 added eq of Mn\(^{2+}\) were incubated with known amounts of Ca\(^{2+}\) between 0 and 2400 eq for one additional hour. Mn\(^{2+}\) and Ca\(^{2+}\) ions were added from stock solutions of their chlorides.

**Oxygen Evolution Measurements**—Steady state PS II enzyme activity at 25 °C was determined by polarographic measurement of the O\(_2\) concentration in a PS II-containing assay mixture using a Clark-type Hansatech oxygen electrode with a high sensitivity Teflon membrane under continuous illumination with a tungsten lamp through an aqueous 5% CuSO\(_4\) IR filter. The assay medium was the buffer of the samples lacking EDTA and with 5 mM MgCl\(_2\) and 0.2 mM phenyl-p-benzoquinone (20 mM in dimethyl sulfoxide) added as an electron acceptor.

**EPR/ENDOR Spectroscopy**—X-band CW EPR spectra were recorded on a Bruker ELEXSYS E500 spectrometer equipped with an ESR900 liquid helium flow cryostat and an ITC503 helium flow temperature controller (Oxford Instruments Ltd.). X-band pulse experiments were performed with a Bruker ESP
Electronic Structure of the Ca\(^{2+}\)-depleted OEC of Photosystem II

380E spectrometer equipped with a dielectric ring resonator, an Oxford ITC liquid helium flow system, and a temperature controller. Q-band pulse experiments were performed using a Bruker ELEXSYS E580 spectrometer equipped with a laboratory-built cylindrical ENDOR resonator (58), a CF935 cryostat, and an ITC5025 temperature controller (Oxford Instruments Ltd.). Field-swept electron spin echo (ESE)-detected experiments were performed at Q-band frequencies using the pulse sequence $\pi/2-\tau-\pi/2-\tau$-echo with $\pi = 72$ ns and $\tau = 440$ ns. For $^{55}$Mn Davers ENDOR, the pulse sequence was $\pi-\pi_{RF}-T-\pi/2-\tau-\pi/2-\tau$-echo, with $\pi = 12$ ns (X-band), 72 ns (Q-band), or 16 ns (Q-band Mn\(^{2+}\) titration/quantification), $\pi_{RF} = 4$ $\mu$s (X-, Q-band) or 4.5 $\mu$s (Q-band Mn\(^{2+}\) titration/quantification), $T = 3.4$ $\mu$s (X-, Q-band) or 1 $\mu$s (Q-band Mn\(^{2+}\) titration/quantification), and $\tau = 200$ ns (X-band), 440 ns (Q-band), or 320 ns (Q-band Mn\(^{2+}\) titration/quantification). The radio frequency (RF) was varied randomly in the desired range, and the RF pulses were amplified by an ENI S100L amplifier. Except for Mn\(^{2+}\) titration/quantification, $^{55}$Mn Davers ENDOR spectra were collected using a home-built computer console with Specman control software (59) coupled to an SMT02 external RF pulse generator.

**EPR/ENDOR Spectral Simulations**—Simulations of EPR and $^{55}$Mn ENDOR spectra were performed numerically using the EasySpin software package (60). The fitting procedures employed a least squares minimization routine. All tensors were set to be collinear. The Ca\(^{2+}\)-depleted Mn\(_4\)O\(_5\) cluster in the S\(_2\)' state was treated as an effective electronic spin $S = 1/2$ ground state coupled to the four $^{55}$Mn nuclei, described by the following spin Hamiltonians for the EPR (Equation 1) and $^{55}$Mn ENDOR (Equation 2) spectra.

$$H_{\text{Mn, O}_{5}\text{EPR} } = \beta_B B_0 GS + \sum_{j=1}^{4} (SA_j)$$

(Eq. 1)

$$H_{\text{Mn, O}_{5}\text{ENDOR} } = \beta_B B_0 GS + \sum_{j=1}^{4} (\beta_B B_0 g_iJ_{ij} + SA_i)$$

(Eq. 2)

The EPR spectrum was calculated using second order perturbation theory, neglecting nuclear Zeeman terms and forbidden transitions. The $^{55}$Mn ENDOR spectra were calculated exactly, including nuclear Zeeman terms and considering all transitions. For the monomeric Mn\(^{2+}\) ion ($S = 5/2, I = 5/2$) bound to the Ca\(^{2+}\)-depleted PS II, the following spin Hamiltonian was solved exactly for the ESE and ENDOR spectra:

$$H_{\text{Mn}^{2+}} = \beta_B B_0 GS + \left[ S_z^2 - \frac{1}{3} S(S + 1) \right] + E(S_x^2 - S_y^2) + \beta_B B_0 g_iJ_i + SA_i$$

(Eq. 3)

For details on the simulation procedure and the theoretical background, see Refs. 46, 49, and 61.

**Temperature-dependent CW EPR Signal Intensity**—The temperature was calibrated using a thermometer in place of the sample in the EPR tube. To guarantee that the actual unsaturated intensity $I_1$ of the S\(_2\)' state modified multiline, as the ground state signal, was measured at all temperatures, the saturation behavior was studied at the lowest temperature employed. As a result, the non-saturating microwave (MW) power of 0.1 milliwatt was used throughout. The intensities $I_1$ of the derivative signals were measured by means of the heights of 19 peaks throughout the spectral range, thereby minimizing statistical errors and contributions of underlying broader signals, such as from cytochrome b\(_{553}\) and the semiquinone-iron complex. How the ground-to-first excited state energy difference $\Delta$ is determined from the temperature dependence of $I_1$ is outlined in the supplemental data.

**Quantification of the Relative Concentrations of PS II-bound Mn\(^{2+}\) and Hexaquo-Mn\(^{2+}\)—**The Mn\(^{2+}\) species in Ca\(^{2+}\) and Mn\(^{2+}\) titration samples were quantified by means of their Q-band $^{55}$Mn Davers ENDOR spectra in two ways, and the results were averaged. (i) The relative contributions of the spectra from the pure Mn\(^{2+}\) species needed to reproduce the spectra from the various titration points were determined. The spectra from Mn\(^{2+}\) already present in the Ca\(^{2+}\)-depleted PS II samples without the addition of Mn\(^{2+}\) ions and from 40 $\mu$M MnCl\(_2\) dissolved in the titration buffer represented PS II-bound and hexaquo-Mn\(^{2+}\), respectively. (ii) The relative amplitudes of the $^{55}$Mn ENDOR $m_3 = -3/2$ transitions, which appear in different RF ranges characteristic for the two Mn\(^{2+}\) species, were quantified in the regions of 353–376 MHz for PS II-bound Mn\(^{2+}\) and 390–395 MHz for hexaquo-Mn\(^{2+}\).

**RESULTS**

**EPR and $^{55}$Mn ENDOR of the Ca\(^{2+}\)-depleted Mn\(_4\)O\(_5\) Cluster in the S\(_2\)' State**—The characteristic modified multiline CW EPR signal (24, 25) was observed for Ca\(^{2+}\)-depleted PS II samples poised in the S\(_2\)' state. It is centered at $g = 2$ and spans the magnetic field range from $\sim 260$ to $\sim 430$ mT, resolving at least 27 hyperfine interaction (HFI) lines with an average peak-to-peak spacing of $\sim 6$ mT (Fig. 2A). The central HFI lines are superimposed by the signal of the stable tyrosyl radical Y\(_D\) centered at $g = 2$, which is not depicted for clarity of presentation. The broad underlying signal of the reduced QA\(_\lambda\)–Fe\(^{2+}\) complex (62) contributes in the 350–375–mT region (24, 25, 29).

**Traces a and b in Fig. 2B** show the X- and Q-band Davies ENDOR spectra of the S\(_2\)' state recorded at 5 K and magnetic fields of 380 and 1208 mT, respectively. The $^{55}$Mn ENDOR spectrum of the Mn\(_4\)O\(_5\) cluster in the S\(_2\)' state is essentially invariant across the corresponding EPR signal envelope (supplemental Fig. S1). It is $\sim 130$ MHz wide, extending over a range from $\sim 60$ to $\sim 190$ MHz. As compared with the $^{55}$Mn ENDOR spectrum of the native S\(_2\) state (Fig. 2Bc, supplemental Fig. S1), the Ca\(^{2+}\)-depleted S\(_2\)' state spectrum is broader. The edges of the spectrum change up to 10 MHz, resulting in a $\sim 20$ and $\sim 10$ MHz increase in the width of the X- and Q-band $^{55}$Mn ENDOR spectra, respectively, as compared with the Ca\(^{2+}\)-containing S\(_2\) state of spinach PS II (42, 45, 46). The Q-band spectrum of the S\(_2\)' state exhibits five clearly resolved peaks, as also seen for the native S\(_2\) state spectrum from Thermosynechococcus elongatus (Fig. 2Bd); however, their positions differ slightly.

The X-band CW EPR and X- and Q-band $^{55}$Mn Davers ENDOR spectra were simultaneously simulated using the spin Hamiltonian formalism (for details see “Experimental Proce-
TABLE 2

Isotropic and anisotropic values of the effective $^{55}\text{Mn}$ HFI tensors $A_i$ ($i = 1–4$) used for the simulations of the X- and Q-band EPR and ENDOR spectra of the Ca$^{2+}$-depleted PS II from spinach in the S$_2$' state (Fig. 2) and for the S$_2$ states of native spinach PS II (46) and native and Sr$^{2+}$-substituted PS II from T. elongatus (49).

<table>
<thead>
<tr>
<th>Species</th>
<th>OEC state</th>
<th>Tensor component</th>
<th>$A_1$</th>
<th>$A_2$</th>
<th>$A_3$</th>
<th>$A_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach</td>
<td>-Ca$^{2+}$ S$_2$'</td>
<td>iso$^a$</td>
<td>311</td>
<td>234</td>
<td>202</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aniso$^b$</td>
<td>72</td>
<td>-84</td>
<td>-38</td>
<td>-59</td>
</tr>
<tr>
<td>Ca$^{2+}$ S$_2$</td>
<td>iso</td>
<td>298</td>
<td>248</td>
<td>205</td>
<td>193</td>
<td>193</td>
</tr>
<tr>
<td>T. elongatus</td>
<td>Ca$^{2+}$ S$_2$</td>
<td>iso</td>
<td>35</td>
<td>-40</td>
<td>-60</td>
<td>-70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aniso</td>
<td>312</td>
<td>251</td>
<td>208</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>Sr$^{2+}$ S$_2$</td>
<td>iso</td>
<td>55</td>
<td>-40</td>
<td>-48</td>
<td>-108</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aniso</td>
<td>332</td>
<td>243</td>
<td>203</td>
<td>173</td>
</tr>
</tbody>
</table>

$^a$ The isotropic $A_{\text{iso}}$ ($i = 1–4$) values are the averages of the principal values: $A_{\text{iso}} = (A_{1x} + A_{1y} + A_{1z})/3$.

$^b$ The anisotropy in the $A_i$ values is expressed as the difference $A_{\text{aniso}} = A_{1z} - A_1$ between the equatorial and axial components of the tensor. The equatorial and axial $A_i$ values are defined as $A_{\text{eq}} = (A_{1x} + A_{1y})/2, A_{\text{axi}} = A_{1z}$.

Spin State Energies of the Ca$^{2+}$-depleted Mn$_4$O$_5$ Cluster in the S$_2$' State—The energy difference $\Delta$ of the paramagnetic ground spin state and the first excited state was estimated from the temperature dependence of the unsaturated X-band CW modified multiline signal of the Ca$^{2+}$-depleted S$_2$' state. The measured intensities $I_1$ of the derivative signal at a series of temperatures are depicted in a Curie plot versus $1/T$ in Fig. 3. This relation is approximately linear over the measured range from 14.4 to 5.5 K and extrapolates to 0 for $T \rightarrow \infty$. Our Curie behavior of the temperature dependence indicates that the Ca$^{2+}$-depleted S$_2$' state features an $S = 1/2$ ground spin state energetically well separated from states of higher spin multiplicity. The temperature dependence of the S$_2$' modified multiline signal can be reproduced reasonably well with $\Delta \approx 35$ cm$^{-1}$ corresponding to $I_{\text{eff}} \approx 12$ cm$^{-1}$ (see "Experimental Procedures"). This relatively large separation from states of higher spin multiplicity allows the S$_2$' state Mn$_4$O$_5$ spin system to be treated in the strong exchange limit, i.e. as an effective $S = 1/2$ spin state, as assumed in the previous section.

EPR and $^{55}\text{Mn}$ ENDOR of a Specifically Bound Mn$^{2+}$ Ion—The Ca$^{2+}$-depleted PS II preparations exhibit an additional EPR and ENDOR signal in all accessible S$^\prime$ states that is not present in native PS II samples. At 5 K, Q-band ESE-detected field sweep EPR spectra of the dark-adapted Ca$^{2+}$-depleted PS II preparations (S$^\prime$-state), in which the Mn$_4$O$_5$ cluster does not show a perpendicular mode EPR signal, displayed a broad EPR signal centered at $g = 1.99$ with a full width at half-maximum of $\sim 63$ mT (Fig. 4, inset). A corresponding signal was not observed using CW X-band EPR spectroscopy; the signal is probably too broad to be discerned from the base-line drift in the CW EPR experiment (51). Q-band Davies ENDOR spectra were recorded at several magnetic fields in the RF frequency range of 30 to 400 MHz (Fig. 4). The $^{55}\text{Mn}$ ENDOR spectra are dominated by two broad peaks between 100–195 MHz and another line centered at $\sim 370$ MHz. The two lines at 100–195 MHz are

FIGURE 3. Curie plot showing the dependence of the intensity $I_1$ of the modified multiline derivative signal of the Ca$^{2+}$-depleted S$_2$' state on the inverse temperature $1/T$. The error of the $x$-values comes from the calibration of the actual temperature at the sample position (see "Experimental Procedures"). The curves are simulations of the Curie temperature dependence over a range of $\Delta$ values on the basis of Equation S1 in the supplemental data and the simplified electron 2-spin coupling scheme for the OEC outlined under "Experimental Procedures." Experimental parameters: MW frequency, 9.437 GHz; MW power, 0.1 milliwatt; modulation amplitude, 0.75 mT; time constant, 82 ms; temperatures, 5.5, 6.3, 7.3, 8.7, and 14.4 K.
dependent on the magnetic field and shift to higher frequencies with increasing magnetic field. The spectra also contain sharp proton signals, one centered at the $^1H$ Larmor frequency (≈50 MHz) and a strongly coupled one at ≈75 MHz with decreasing amplitude at increasing field positions. Its partner at low frequency (∼25 MHz) lies outside the spectral range. No further ENDOR signals were detected for this species using either ENDOR or electron spin echo envelope modulation (ESEEM).

These EPR and $^{55}$Mn ENDOR signals can be readily assigned to high spin Mn$^{2+}$ with $S = 5/2$, although their appearance is different from the spectra typically associated with Mn$^{2+}$ complexes (see Discussion and Fig. 5A). Simultaneous simulations of the EPR and of four ENDOR spectra at different magnetic fields (Fig. 4, dashed red traces) are consistent with this assignment. They reproduce both the spectral breadth and line shape of the EPR absorption signal and the peaks in the four $^{55}$Mn ENDOR spectra. Besides a near-isotropic average $G_{iso}$ of 258 MHz, the simulations yielded an almost iso of 258 MHz, and a strongly coupled one at ≈75 MHz with decreasing amplitude at increasing field positions. Its partner at low frequency (∼25 MHz) lies outside the spectral range. No further low frequency signals were detected for this species using either ENDOR or electron spin echo envelope modulation (ESEEM).

Simultaneous simulations of the EPR and of four ENDOR spectra at different magnetic fields (Fig. 4, dashed red traces) are consistent with this assignment. They reproduce both the spectral breadth and line shape of the EPR absorption signal and the peaks in the four $^{55}$Mn ENDOR spectra. Besides a near-isotropic average $G_{iso}$ of 258 MHz, the simulations yielded an almost iso of 258 MHz, and a strongly coupled one at ≈75 MHz with decreasing amplitude at increasing field positions. Its partner at low frequency (∼25 MHz) lies outside the spectral range. No further low frequency signals were detected for this species using either ENDOR or electron spin echo envelope modulation (ESEEM).

Simultaneous simulations of the EPR and of four ENDOR spectra at different magnetic fields (Fig. 4, dashed red traces) are consistent with this assignment. They reproduce both the spectral breadth and line shape of the EPR absorption signal and the peaks in the four $^{55}$Mn ENDOR spectra. Besides a near-isotropic average $G_{iso}$ of 258 MHz, the simulations yielded an almost iso of 258 MHz, and a strongly coupled one at ≈75 MHz with decreasing amplitude at increasing field positions. Its partner at low frequency (∼25 MHz) lies outside the spectral range. No further low frequency signals were detected for this species using either ENDOR or electron spin echo envelope modulation (ESEEM).

Simultaneous simulations of the EPR and of four ENDOR spectra at different magnetic fields (Fig. 4, dashed red traces) are consistent with this assignment. They reproduce both the spectral breadth and line shape of the EPR absorption signal and the peaks in the four $^{55}$Mn ENDOR spectra. Besides a near-isotropic average $G_{iso}$ of 258 MHz, the simulations yielded an almost iso of 258 MHz, and a strongly coupled one at ≈75 MHz with decreasing amplitude at increasing field positions. Its partner at low frequency (∼25 MHz) lies outside the spectral range. No further low frequency signals were detected for this species using either ENDOR or electron spin echo envelope modulation (ESEEM).
were performed, monitoring the CW EPR and ENDOR signal described above.

**Mn^{2+}/Ca^{2+} Titration Monitored by CW EPR—Mn^{2+} ions were added to Ca^{2+}-depleted PS II samples and the characteristic $S_2$, $Y'_Z$, state split signal, $S'_Z$ multiligne signal, and hexaquo-Mn^{2+} signal (not shown) were measured. The addition of $ \leq 0.8$ eq of Mn^{2+} ions relative to the number of PS II RCs did not quantitatively alter the three signals. The Mn^{2+} ions added are CW EPR-silent, as seen in the study of Booth et al. (51), which is consistent with a protein-bound Mn^{2+} species. In addition, this species does not cause any line broadening or even splitting of the signals from the OEC or the tyrosyl radicals. The addition of $ \geq 0.8$ equivalents of Mn^{2+} ions resulted in the appearance of the hexaquo-Mn^{2+} signal. The subsequent addition of Ca^{2+} to Ca^{2+}-depleted, Mn^{2+}-loaded PS II samples led to a loss of the $S'_2$, $Y'_Z$ state split signal and of the multiline signal, as the Ca^{2+}-reconstituted Mn$_4$O$_5$Ca cluster can proceed beyond the $S'_2$, state upon illumination. A concomitant increase of the Mn^{2+} six-line signal was observed due to the release of the PS II-bound Mn^{2+} into solution (51).

**Mn^{2+}/Ca^{2+} Titration Monitored by $^{55}$Mn ENDOR—Mn^{2+} binding was also directly monitored by Q-band ENDOR. The concentrations of PS II-bound and solubilized Mn^{2+} ions in each sample were quantified by means of the relative amplitudes of their characteristic $^{55}$Mn ENDOR signals (Fig. 5A; for the titration curve, see supplemental Fig. S3). Without the addition of MnCl$_2$, dark-adapted Ca^{2+}-depleted PS II ($S'_1$, state) always displayed the PS II-bound Mn^{2+} signal shown in Fig. S4. The addition of $ \sim 0.8$ eq of MnCl$_2$ led to a 4–5-fold increase of this signal with only little free hexaquo-Mn^{2+} (15 ± 4%) present at the same time. This suggests that $ \sim 20\%$ of RCs contain a bound Mn^{2+} before exogenous addition of MnCl$_2$ so that in the end a total of $ \sim 1$ eq Mn^{2+} is in the sample. The basal Mn^{2+} is likely derived from centers damaged during the Ca^{2+} depletion procedure and nominally corresponds to the loss of $ \sim 5\%$ Mn$_4$O$_5$(Ca) clusters. The high occupancy of the Mn^{2+} site suggests that it is of high affinity, with a dissociation constant $K_D$ that is too small to be determined here. From the employed concentrations of the binding partner, $K_D$ is expected to be in the submicromolar/nanomolar range. It is also noted that the addition of the chelating agent EDTA did not remove or alter the appearance of the bound Mn^{2+} signal, consistent with the protein site having a high affinity for Mn^{2+}.

An additional Ca^{2+} titration was performed on the fully Mn^{2+}-loaded Ca^{2+}-depleted PS II (+0.8 eq of MnCl$_2$, i.e. a final ratio of 1 Mn^{2+} ion per PS II RC). The Ca^{2+} concentrations ranged from 0 to 2400 eq Ca^{2+} per RC (0–60 mM). In Fig. 5B, the relative concentrations of the two Mn^{2+} species (PS II-bound and solubilized) are plotted against the equivalents of Ca^{2+} ions added. This behavior could be reproduced by a sigmoid curve with a half-saturation value of 700 Ca^{2+} ions per RC. This value is similar to 1200 eq of Ca^{2+} reported in Booth et al. (51). The difference may be due to the Ca^{2+} depletion method used, the low pH/citrate treatment in this study versus a NaCl salt wash (24) in the study of Booth et al. (51). Their differing effects on the extrinsic PS II subunit composition could alter the Ca^{2+} binding kinetics (see Refs. 24 and 51).

**DISCUSSION**

**Location of the Mn^{2+} Binding Site—**Based on the observations described above (see “Results”), a preliminary assignment can be made as to where the binding site of the Mn^{2+} ion is located. No strong magnetic interaction was observed between the Mn^{2+} ion and the Ca^{2+}-depleted Mn$_4$O$_5$ cluster or the tyrosyl radical $Y'_Z$ in the form of a broadening or splitting of the corresponding EPR signals. Thus, Mn^{2+} binding directly to the Ca^{2+} site of the OEC can be excluded. This Mn^{2+} ion must be at least 10 Å away not to be detectable via dipolar magnetic interaction. A similar argument holds for $Y'_D$ (D2-Tyr-160), as it also displays an unperturbed EPR lineshape when Mn^{2+} is bound. These “exclusion zones” are indicated by green and violet spheres in Fig. 6A. There is, however, a long range dipolar interaction between the Mn^{2+} ion and $Y'_D$, as evidenced by the relaxation enhancement of its EPR signal (51). Being smaller than the enhancement resulting from the Mn$_4$O$_5$Ca cluster in the $S'_2$ state suggests a weaker Mn^{2+}–$Y'_D$ interaction and thus a longer distance than the 31 Å measured between the cluster and $Y'_D$ (6).

The binding and titration behavior can either be rationalized by a significant allosteric effect of Ca^{2+} on the Mn^{2+} site, or Mn^{2+} binding could take place directly at a depleted Ca^{2+} site. The recent crystal structure (6) of PS II from Thermosynechococcus vulcanus exhibits three additional Ca^{2+} sites at distances greater than 30 Å from the paramagnetic species monitored, i.e. the Mn$_4$O$_5$(Ca) cluster, $Y'_Z$, and $Y'_D$ (64A). In the structure of PS II from T. elongatus, a different Ca^{2+} site in PsbO has been identified (4, 5, 68), not found in the T. vulcanus crystals. All these Ca^{2+} sites are located on the luminal/donor side of PS II in the subunit CP47, the cytochrome b$_{599}$ subunit β (PsbF), and the extrinsic protein PsbO, and are solvent-accessible. It is not clear, however, whether Ca^{2+} binding at these sites is solely a crystallization artifact under the conditions used or of physiologically relevance. With the exception of the two sites in PsbO, the Ca^{2+} sites appear to be of low affinity, as the Ca^{2+} ions are ligated to a large part by H$_2$O and glycerol. In contrast, the two Ca^{2+} sites seen in the PsbO possess at least three ligands from amino acid side chains (Fig. 6, B and C) and thus are potentially of high affinity. In the homologous PsbO from spinach, which has also been reported to bind Ca^{2+} (69–71), Asn-197 and Val-198 of the binding motif in Fig. 6B correspond to the conserved residues Ser-286 and Val-287, whereas there is no equivalent for Thr-135, Glu-81, Glu-140, and His-257 in the other binding motif (Fig. 6C) correspond to Glu-146, Glu-205, and Glu-317 (for a sequence alignment, see supplemental Fig. S4). Mn^{2+} binding to PsbO has indeed been demonstrated previously in isolated PsbO from higher plants (72–74). As in the present study and Ref. 51, protein-bound Mn^{2+} did not show a CW EPR signal, but a six-line signal was observed after denaturation of the protein (73). PsbO was reported to show carbonic anhydrase activity, which was maximal in the presence of Mn^{2+} (74).

The magnetic properties of the Mn^{2+} ion provide information about the immediate ligand environment in this binding pocket. The $D$ and $E$ values of Mn^{2+} complexes of higher symmetry, such as Mn^{2+}-EDTA and hexaquo-Mn^{2+}, are significantly smaller than those for the PS II-bound Mn^{2+} described.
Electronic Structure of the Ca$^{2+}$-depleted OEC of Photosystem II

The physiological role of the putative Mn$^{2+}$ binding site has been found to bind at one of the two proposed Mn$^{2+}$ sites in PsbP crystals from spinach (PDB accession number 3ARC) (6) (PDB accession number 3ARC) highlighting the Ca$^{2+}$ ions (black spheres) as well as a Ca$^{2+}$ binding site found in PS II from T. elongatus (gray sphere) and their distances to the paramagnetic entities Mn$_i$O$_j$Ca cluster, Y$_{i/2}$, and Y$_{i'}$. The 10 Å spheres around the latter indicate the approximate region in which a bound Mn$^{2+}$ would cause a splitting of their EPR signals and thus can be excluded to contain the Mn$^{2+}$ binding site. B and C, ligand environments of the Ca$^{2+}$ ions in the extrinsic PsbO proteins from T. vulcanus and T. elongatus (T.e.) (5), respectively. Oxygen, nitrogen, and carbon atoms are shown in red, blue, and yellow, respectively. Differences between the PsbO proteins of these cyanobacterial species and from higher plant spinach are displayed by a sequence alignment in supplemental Fig. S4. All distances are in Å.

FIGURE 6. Ca$^{2+}$ and potential Mn$^{2+}$ binding sites in cyanobacterial PS II crystals. A, PS II crystal structure from T. vulcanus (6) (PDB accession number 3ARC) highlighting the Ca$^{2+}$ ions (black spheres) as well as a Ca$^{2+}$ binding site found in PS II from T. elongatus (gray sphere) and their distances to the paramagnetic entities Mn$_i$O$_j$Ca cluster, Y$_{i/2}$, and Y$_{i'}$. The 10 Å spheres around the latter indicate the approximate region in which a bound Mn$^{2+}$ would cause a splitting of their EPR signals and thus can be excluded to contain the Mn$^{2+}$ binding site. B and C, ligand environments of the Ca$^{2+}$ ions in the extrinsic PsbO proteins from T. vulcanus and T. elongatus (T.e.) (5), respectively. Oxygen, nitrogen, and carbon atoms are shown in red, blue, and yellow, respectively. Differences between the PsbO proteins of these cyanobacterial species and from higher plant spinach are displayed by a sequence alignment in supplemental Fig. S4. All distances are in Å.
Electronic Structure of the Ca$^{2+}$-depleted OEC of Photosystem II

TABLE 3
Calculated spin projection tensor components $\rho_{ij}$ and $\rho_{ij}$, intrinsic $^{55}$Mn HFI tensor components $a_{ij}$ and $a_{ij}$, and isotropic and anisotropic intrinsic HFI values $a_{iso}$ and $a_{aniso}$ for the Mn ions of the OEC in the Ca$^{2+}$-depleted $S'_2$ state on the basis of the electronic exchange-coupling scheme in Fig. 7 with $c = 1.65$ and intrinsic ZFS values $d_{iso} = d_{3} = c_{2} = 0$ cm$^{-1}$ for the Mn$^{IV}$ ions and $d_{2} = -2.27$ cm$^{-1}$ for the Mn$^{III}$ ion.

<table>
<thead>
<tr>
<th>Manganese ion</th>
<th>$\rho_{ij}$</th>
<th>$\rho_{ij}$</th>
<th>$a_{ij}$</th>
<th>$a_{ij}$</th>
<th>$a_{iso}$</th>
<th>$a_{aniso}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn$^{IV}$</td>
<td>MHz</td>
<td>MHz</td>
<td>MHz</td>
<td>MHz</td>
<td>MHz</td>
<td>MHz</td>
</tr>
<tr>
<td>Mn$_{A4}$ ($^{55}$Mn)</td>
<td>1.03 1.25</td>
<td>197 230</td>
<td>208 33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn$_{A2}$ ($^{55}$Mn)</td>
<td>-0.81 -1.09</td>
<td>187 190</td>
<td>188 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn$_{C2}$ ($^{55}$Mn)</td>
<td>-0.87 -1.21</td>
<td>220 188</td>
<td>209 -31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn$_{D1}$ ($^{55}$Mn)</td>
<td>1.66 2.04</td>
<td>202 123</td>
<td>175 -79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn$_{D1}$ ($^{55}$Mn)</td>
<td>0.75 1.05</td>
<td>152 188</td>
<td>206 33</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The equatorial and axial $a_{ij}$ values are defined as $a_{ij} = (a_{ab} + a_{ac})/2$, $a_{ij} = a_{ij}$.
* The isotropic $a_{iso}$ values are the averages of the individual components of the tensor $a_{iso} = (a_{ab} + a_{ac} + a_{ij})/3$.
* The anisotropy of the $a$ tensor is expressed as the difference $a_{aniso} = a_{ij} - a_{ij}$ between the parallel and perpendicular tensor components.

ESE and ENDOR spectra (not shown). It is emphasized though that this effect is of the same size as that of the variation between species and thus is unlikely to be of physiological significance.

Electronic Structure/Exchange Coupling Scheme of the Ca$^{2+}$-depleted Mn$_4$O$_5$ Cluster in the $S'_2$ State—To further rationalize the spectral results from the Ca$^{2+}$-depleted Mn$_4$O$_5$ cluster, a spin coupling scheme for the $S'_2$ state was developed. It was constructed to meet the following requirements: (i) a ground state of spin multiplicity $S = 1/2$, (ii) a ground-to-first excited state energy difference $\Delta \approx 35$ cm$^{-1}$, (iii) spin projection factors $|\rho_{ij}| \approx 1$ for all four manganese electronic spins, and (iv) intrinsic ZFS constants $d_{ij}$ of the manganese ions that lie within the range found for mono- and dimeric model complexes, i.e. $1$ cm$^{-1} < |d_{ij}| < 5$ cm$^{-1}$ for Mn$^{III}$ and $|d_{ij}| < 0.1$ cm$^{-1}$ for Mn$^{IV}$ ions in an octahedral ligand environment (see Refs. 18, 47, and 49). The inferred structural (29) and spectral similarity of the native and the Ca$^{2+}$-depleted manganese cluster suggest that the spin coupling scheme for the native $S_2$ state (Fig. 7, $c = 1$) (18), in which Mn$_{D1}$ is the Mn$^{III}$ ion, can be used as a starting point. Calculated on the basis of the refined model of the OEC in the latest crystal structure (6), the basic arrangement of this scheme is in accordance with the spatial organization as described by Siegbahn and our group (17, 18, 47, 85), in which Mn$_{B3}$, Mn$_{C2}$, and Mn$_{D1}$ form a trimeric core unit connected to Mn$_{A4}$ by a di-$\mu$-oxo bridge via Mn$_{B3}$ (Fig. 1). Thus, this scheme represents an extension of the (3 + 1)- or Y-coupling schemes, proposed earlier in EPR spectroscopic studies (42, 46, 47, 49), where $J_{A4-B3} = J_{A4-D1} = 0$.

The coupling topology fulfills criteria (i) and (iii) as ground spin state multiplicity and spin projection factors are the same for the two states, $S_2$ and $S'_2$. In contrast, their ground-to-first excited state energy differences $\Delta$ and effective $^{55}$Mn HFI tensors $A_{ij}$, relevant for (ii) and (iv), are different. Thus, the $\Delta = 10.5$ cm$^{-1}$ calculated for the $S_2$ state coupling scheme also differs from the experimental $\Delta \approx 35$ cm$^{-1}$ determined for the $S'_2$ state. Correlations between the exchange coupling scheme and this energy difference have been investigated in previous studies (41, 47). One mechanism by which $\Delta$ is influenced directly is shown to be the strength and the sign of the exchange coupling between Mn$_{A4}$ and the trimeric unit comprising Mn$_{B3}$, Mn$_{C2}$, and Mn$_{D1}$. An increase or decrease in the magnitudes of the coupling constant $J_{A4-B3}$ results in a larger or smaller energy gap, respectively. As the monomer-trimer joint is in the vicinity of a possible binding site of a MeOH molecule, it rationalizes the effect of MeOH on the electronic structure of the Mn$_4$O$_5$Ca cluster in the native $S_2$ state (41). For the Ca$^{2+}$-depleted $S'_2$ state, the coupling of Mn$_{A4}$ to the trimeric unit was varied by multiplying the respective exchange coupling constants $J_{A4-B3}/J_{A4-C2}$ and $J_{A4-C2}$ by a factor $c$ (Fig. 7). It can be readily calculated that with $c = 1.65$ ($J_{A4-B3} = -46$ cm$^{-1}$, $J_{A4-C2} = 7$ cm$^{-1}$, and $J_{A4-D1} = 10$ cm$^{-1}$), $\Delta$ is 35 cm$^{-1}$ and thus in the desired range.

For testing whether the obtained model also reproduces reasonable estimates for the intrinsic ZFS values $d_{ij}$ of the Mn$^{III}$ and Mn$^{IV}$ ions, a brief description on how those can be assessed based on the inferred coupling scheme and the fitted effective HFI tensors is given in the supplemental data. Because of their inherently small ZFFs, the $d_{ij}$ values of the three Mn$^{IV}$ ions can be assumed to be $0$ cm$^{-1}$ for the calculations of the intrinsic HFI tensors $a_{ij}$ from the fitted effective $A_{ij}$ and the computed $\rho_{ij}$ tensors. Mn$^{III}$ ions generally exhibit an absolute isotropic HFI value $|a_{iso}|$ in the range between 165 and 225 MHz and considerable anisotropy defined as the difference $a_{aniso} = |d_{ij}| - |a_{ij}|$ between the absolute values. Mn$^{IV}$ ions tend to exhibit slightly larger isotropic HFI values ($|d_{iso}| = 187–253$ MHz) and only small intrinsic HFI anisotropies ($|a_{iso}| < 30$ MHz) (see Ref. 49). For the Ca$^{2+}$-depleted $S'_2$ state, a ZFS value $d_{iso}$ of the Mn$_{D1}$ ion in the range of $-2.24$ to $-2.31$ cm$^{-1}$ yields $a_{ij}$ tensors consistent with the valence states of the individual manganese ions. An optimized ZFS value $d_{iso} = -2.27$ cm$^{-1}$ leads to the spin projection and intrinsic HFI tensors $\rho_{ij}$ and $a_{ij}$ listed in Table 3. In terms of the intrinsic isotropic and anisotropic HFI values, the calculated numbers match the prerequisites as found in the literature very well. As the ZFS $d_{iso} = -2.24$ to $-2.31$ cm$^{-1}$ lies in the range usually found for Mn$^{III}$ ions (1 cm$^{-1} < |d_{ij}| < 5$ cm$^{-1}$), the developed model fulfills the four essential criteria imposed.

Structural Implications of the Zero-field Splitting $d_{D1}$ of the Mn$_{D1}$ $^{III}$ Ion—The removal of the Ca$^{2+}$ ion from the spinach OEC is found to result in a significant change of $d_{D1}$ from $-1.2$ cm$^{-1}$ (41) to $-2.2$ to $-2.3$ cm$^{-1}$. This perturbation is larger than for the Ca$^{2+}$/Sr$^{2+}$ replacement in PS II from T. elongatus. For these systems, the intrinsic ZFS values of the Mn$_{D1}$ $^{III}$ ion are relatively similar (Ca$^{2+}$, $d_{D1} = -1.3$ cm$^{-1}$; Sr$^{2+}$, $d_{D1} = -1.4$ cm$^{-1}$). (caused by the removal of the Ca$^{2+}$ ion from the spinach OEC is found to result in a significant change of $d_{D1}$ from $-1.2$ cm$^{-1}$ (41) to $-2.2$ to $-2.3$ cm$^{-1}$. This perturbation is larger than for the Ca$^{2+}$/Sr$^{2+}$ replacement in PS II from T. elongatus. For these systems, the intrinsic ZFS values of the Mn$_{D1}$ $^{III}$ ion are relatively similar (Ca$^{2+}$, $d_{D1} = -1.3$ cm$^{-1}$; Sr$^{2+}$, $d_{D1} = -1.4$ cm$^{-1}$).
Electronic Structure of the Ca$^{2+}$-depleted OEC of Photosystem II

![Diagram](image.png)

FIGURE 8. Scheme of the native Mn$_4$O$_5$Ca cluster in the $S_0$ state and the Ca$^{2+}$-depleted $S_{2'}$ state represented by a hypothesized Mn$_4$O$_5$ cluster. In the putative $S_{2'}$ state, the fast exchanging substrate water is already bound to Mn$_{D1}^{III}$, filling the space of the Ca$^{2+}$ ion. $W_i$ and $W_f$ denote the slowly and fast exchanging substrate waters, respectively (96, 99).

$\text{s}^2$ cm$^{-1}$) (49). It is, however, noted that the signs of the $d_{D1}^{III}$ and of the HFI anisotropy of the Mn$_{III}$ ion do not change between the Ca$^{2+}$-depleted $S_2$ and the Ca$^{2+}$-containing $S_2$ state. These parameters can be related to the ligand sphere of the Mn$_{III}$ ion (86–88). Negative numbers for $d_{D1}$ and $a_{D1,aniso}$ correspond to a $^{5}B_6$ ground state, obtained in the cases of square pyramidal 5-coordinate or tetragonally elongated 6-coordinate ligand geometries. This suggests the coordination sphere of the Mn$_{D1}^{III}$ for the $S_2$ and $S_2$ states to be similar. However, the increase in the magnitude of $d_{D1}$ upon Ca$^{2+}$ removal does indicate modifications of the precise binding mode, e.g. altered ligand distances and angles. One possible mechanism for altering $d_{D1}$ is protonation of one of the $\mu$-oxo bridges ligating the Ca$^{2+}$ ion (Fig. 1) as a means of overall charge compensation of the cluster upon Ca$^{2+}$ removal. It is known from model complexes that Mn-Mn distances are elongated upon protonation of Mn-O-Mn bridges (89). However, within the trimeric cuboidal unit, this lengthening could be strongly impaired for the Mn$_{C2}$-Mn$_{D1}$ distance. The fitted averaged distance of the Mn-Mn interactions at 2.7–2.8 Å from EXAFS on Ca$^{2+}$-depleted PS II samples (29), however, does not allow for a conclusive assessment. Also, glutamate 189 of the D1 protein (D1-Glu-189), which directly coordinates the Mn$_{D1}^{III}$ (6, 17, 47) and potentially also the Ca$^{2+}$ ion (18), could be reoriented upon Ca$^{2+}$ depletion leading to a distortion of the coordination sphere and thus an altered $d_{D1}$.

In the latest crystal structure, all four manganese ions are 6-coordinate (6). This, however, requires the O5 $\mu$-oxo bridge to be a ligand of Mn$_{A4}$, Mn$_{B3}$, and Mn$_{D1}$, engendering very long Mn-O5 bond distances well outside the range seen in model complexes (see Ref. 18) and by EXAFS spectroscopy of the Mn$_4$O$_5$Ca cluster in PS II (90, 91). In most geometry-optimized DFT structures, such as those proposed by Siegbahn and our group (17, 18, 47), the position of O5 is significantly altered (Fig. 1). The O5 shifts toward the Mn$_{A4}$, forming a genuine $\mu$-oxo bridge between Mn$_{A4}$ and Mn$_{B3}$ and results in Mn$_{D1}$ having an open coordination site. In this case, in the Ca$^{2+}$-depleted $S_{2'}$ state, Glu-189 might function as a bidentate ligand in a then tetragonally elongated 6-coordinate Mn$_{D1}^{III}$ ligand sphere, leading to the observed change of $d_{D1}$.

Alternatively, the absence of Ca$^{2+}$ may allow this open site to be occupied by a water molecule in the $S_{2'}$ state (Fig. 8) forming a sixth ligand to Mn$_{D1}$. The Mn$_{D1}$-bound water molecule is the second substrate water in the mechanism proposed by Sieg-bahn (17), which potentially binds during the $S_2$-to-$S_1$ transition. Thus, within this model, one of the roles of Ca$^{2+}$ in the active cluster would be to prevent the second substrate from binding too early in the reaction cycle (25, 92). This activity would presumably avoid detrimental side product formation (reactive oxygen species) and lead to single product (O$_2$) formation. Consistent with this role for the Ca$^{2+}$ ion is the known S state dependence of the affinity of Ca$^{2+}$ to this site (93). It drops significantly in the $S_3$ state, suggesting that in this state Ca$^{2+}$ is less tightly bound, having a more flexible ligand sphere that potentially allows greater solvent access to the Mn$_{D1}$ ion.

Besides $\mu$-oxo bridge protonation, the loss of two positive charges is likely to be compensated by protonation of amino acid residues ligating the Ca$^{2+}$ ion in the intact cluster. Other possibilities are the replacement of Ca$^{2+}$ by monovalent Na$^+$ in the samples or the absence of complete charge compensation, leaving the Mn$_4$O$_5$ cluster with an additional negative charge. It is evident that any of these modifications could have a critical effect on the catalytic capabilities of the cluster, especially with regard to proton-coupled electron transfer to $Y_Z$. In light of the proposed deprotonation sequence 1,0,1,2 for the individual oxidation steps starting from $S_0$ (94), this would explain the Mn$_{D1}$ cluster being able to advance to $S_{2'}$ but not from $S_2$-$Y_{Z'}$ to $S_{4'}$.

Conclusions—This study demonstrates that Ca$^{2+}$ is not required for conferring the critical electronic properties to the Mn$_4$O$_5$Ca cluster. This also confirms that Ca$^{2+}$ is not essential for structural maintenance of the OEC. Its presence or absence does not affect the position of the only Mn$_{III}$ ion of the cluster in the $S_2$/$S_{2'}$ state (Mn$_{D1}$), and the contribution of the four manganese ions to the electronic states $S_2$ and $S_{2'}$ does not differ considerably. Thus, the necessity for Ca$^{2+}$ in water splitting catalysis must be due to another functional role of the Ca$^{2+}$ ion.

Although the exact mechanism of inhibition upon Ca$^{2+}$ removal is still unclear, two models can be considered in terms of the two basic catalytic mechanisms proposed in the literature. (i) For mechanisms that involve O-O bond formation between a Ca$^{2+}$-bound and a manganese-bound substrate water (be it a terminal ligand Mn$_V$ = O or a $\mu$-oxo bridge) (11, 95–97), inhibition due to Ca$^{2+}$ depletion is readily explained. The enzyme is inactive, as it has lost a substrate binding site. It should be noted though that this model provides no rationale for the fact that the catalytic cycle is blocked at the stage of $S_2$-$Y_{Z'}$. (ii) Instead, O-O bond formation has been proposed to follow a mechanism that results in the coupling of substrates bound to two manganese sites (be it between two terminal bound Mn-O ligands or involving a $\mu$-oxo bridge via oxyl radical coupling) (10, 14, 16, 17, 98). Then, inhibition due to Ca$^{2+}$ removal probably represents a secondary effect where the Ca$^{2+}$ ion is critical for maintaining the H-bond network between $Y_{Z}$ and the manganese cluster (6, 11, 30) as opposed (or in addition) to perturbation of substrate binding. Thus, Ca$^{2+}$ removal would disable proton-coupled electron transfer during the $S_2$-$Y_{Z'}$-$S_4$ transition, preventing substrate deprotonation and concomitant oxidation of Mn$_{D1}^{III}$. Therefore, the elucidation of the mechanistic role of the Ca$^{2+}$ ion in the OEC is tightly linked to understanding the mechanism of photosynthetic water splitting.
Electronic Structure of the Ca$^{2+}$-depleted OEC of Photosystem II

REFERENCES

Electronic Structure of the Co^{2+}-depleted OEC of Photosystem II


SUPPLEMENTAL DATA

The Basic Properties of the Electronic Structure of the Oxygen-Evolving Complex of Photosystem II are not Perturbed by Ca$^{2+}$ Removal

Thomas Lohmiller,‡ Nicholas Cox,‡ Ji-Hu Su,‡§ Johannes Messinger,‖ Wolfgang Lubitz‡,1

‡Max-Planck-Institut für Bioanorganische Chemie, Stiftstrasse 34-36, 45470 Mülheim an der Ruhr, Germany.

§Present address: Department of Modern Physics, University of Science and Technology of China, Hefei, Anhui 230026, China.

‖Department of Chemistry, Chemical Biological Centre (KBC), Umeå University, S-90187 Umeå, Sweden.

1To whom correspondence should be addressed: Prof. Dr. Wolfgang Lubitz, Max-Planck-Institut für Bioanorganische Chemie, Stiftstrasse 34-36, 45470 Mülheim an der Ruhr, Germany, Tel: +49 208 306 3614. Fax: +49 208 306 3955. E-mail: wolfgang.lubitz@mpi-mail.mpg.de.
SUPPLEMENTAL FIGURE S1. X-band Davies ENDOR spectra of the Ca\textsuperscript{2+}-depleted OEC poised in the S\textsubscript{2'} state in PS II isolated from spinach at various magnetic fields and, for comparison, of the native OEC in the S\textsubscript{2} state in the presence of 3 % MeOH at \(B_0 = 360\) mT (bottom trace), as published in Refs. (1, 2). The S\textsubscript{2'} state spectra were smoothed using a 9-point moving average. Experimental parameters (S\textsubscript{2'} state): MW frequency: 9.717 GHz; shot repetition rate: 5 μs; MW pulse length \(\pi\): 12 ns; \(\tau\): 200 ns; magnetic fields \(B_0\): 320 mT, 340 mT, 356 mT, 380 mT (from the top); RF pulse length \(\pi_{RF}\): 4 μs; temperature: 5 K; accumulations/time: 460/355 min, 166/149 min, 160/144 min, 339/305 min (from the top).
SUPPLEMENTAL FIGURE S2. Q-band Davies ENDOR spectra of Ca$^{2+}$-depleted PS II isolated from spinach illustrating the subtraction of the contaminating Mn$^{2+}$ signal from the raw data obtained from the sample poised in the S$_{2}'$ state, which yields the pure spectrum of the Ca$^{2+}$-depleted Mn$_4$O$_5$ cluster in the S$_{2}'$ state. Top trace (1): Spectrum of an illuminated sample poised in the S$_{2}'$ state containing both the S$_{2}'$ state signal and contributions from residual Mn$^{2+}$ ions. Middle trace (2): Spectrum of a dark-adapted sample poised in the S$_{1}'$ state before illumination showing only the Mn$^{2+}$ signal. For the subtraction, both spectra (1) and (2) were normalized with respect to the signal around 370 MHz, to which only the Mn$^{2+}$ ion contributes. Bottom trace (1 – 2): difference of the spectra from samples in the S$_{2}'$ and the S$_{1}'$ states, the result of which is the spectrum of the OEC in the S$_{2}'$ state. Experimental settings: MW frequency: 34.033 GHz; shot repetition rate: 5 μs; MW pulse length $\pi$: 72 ns; $\tau$: 480 ns; magnetic field $B_0$: 1208 mT; RF pulse length $\pi_{RF}$: 4 μs; temperature: 5 K; accumulations/time: 202/218 min (1), 358/387 min (2).
SUPPLEMENTAL FIGURE S3. Titration of dark-adapted Ca$^{2+}$-depleted PS II samples (S$_i$ state) with Mn$^{2+}$. The relative Q-band $^{55}$Mn Davies ENDOR signal intensities of Mn$^{2+}$ ions bound to the PS II protein complex (black squares) and hexaquo-Mn$^{2+}$ in solution (red circles), quantified as described in the Experimental Procedures section 2.6 (main text), are plotted against the equivalents of Mn$^{2+}$ ions added to the samples. The concentration of the defined PS II-bound Mn$^{2+}$ species as a function of added Mn$^{2+}$ was reproduced by means of a sigmoid curve fitted to the determined intensities up to 1.2 equivalents of Mn$^{2+}$ in which the zero crossing of the x-axis was shifted to -0.2 equivalents with a half-binding value of 0.47 equivalents (solid line). The increase of hexaquo-Mn$^{2+}$, as well as additionally unspecifically bound hexaquo-Mn$^{2+}$ ions was reproduced by linear fits to the measured intensities above 0.64 equivalents of added Mn$^{2+}$ ions (dashed lines). The concentration of reaction centers in the samples was 25 ± 3 μM. For the experimental parameters of the $^{55}$Mn Davies ENDOR measurements see Fig. 4A (main text).
SUPPLEMENTAL FIGURE S4. Amino acid sequence alignment of the PsbO proteins from the cyanobacteria *T. vulcanus* and *T. elongatus* and the higher plant spinach (*Spinacea oleracea*). Residues ligating the Ca$^{2+}$ ions at the sites identified in the PS II crystal structures from *T. vulcanus* and *T. elongatus* are highlighted in red and blue, respectively. The protein sequence alignment was performed using the BLAST search engine provided by UniProt (3).
**SUPPLEMENTAL TABLE S1.** Principal Values\(^a\) and Isotropic\(^b\) and Anisotropic\(^c\) Values of the Effective \(G\) and \(^{55}\text{Mn}\) HFI Tensors \(A_i\) \((i = 1\text{-}4)\) for the Simulations of the X- and Q-Band EPR and ENDOR Spectra of the \(\text{Ca}^{2+}\)-depleted PS II from Spinach in the \(S_2'\) State (Fig. 2, Main Text) and for the \(S_2\) States of Native Spinach PS II (2) and Native and \(\text{Sr}^{2+}\)-substituted PS II from \(T.\) elongatus (4).

<table>
<thead>
<tr>
<th></th>
<th>(G)</th>
<th>(A_1) / MHz</th>
<th>(A_2) / MHz</th>
<th>(A_3) / MHz</th>
<th>(A_4) / MHz</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spinach</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-(\text{Ca}^{2+}) (S_2')</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x)</td>
<td>1.979</td>
<td>342</td>
<td>212</td>
<td>173</td>
<td>139</td>
</tr>
<tr>
<td>(y)</td>
<td>1.986</td>
<td>328</td>
<td>199</td>
<td>205</td>
<td>164</td>
</tr>
<tr>
<td>(\perp^a)</td>
<td>1.983</td>
<td>335</td>
<td>206</td>
<td>189</td>
<td>152</td>
</tr>
<tr>
<td>(z (|)^a)</td>
<td>1.979</td>
<td>263</td>
<td>290</td>
<td>227</td>
<td>211</td>
</tr>
<tr>
<td>(\text{iso}^b)</td>
<td>1.981</td>
<td>311</td>
<td>234</td>
<td>202</td>
<td>171</td>
</tr>
<tr>
<td>(\text{aniso}^c)</td>
<td>0.004</td>
<td>72</td>
<td>-84</td>
<td>-38</td>
<td>-59</td>
</tr>
<tr>
<td><strong>(\text{Ca}^{2+}) (S_2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x)</td>
<td>1.997</td>
<td>310</td>
<td>235</td>
<td>185</td>
<td>170</td>
</tr>
<tr>
<td>(y)</td>
<td>1.970</td>
<td>310</td>
<td>235</td>
<td>185</td>
<td>170</td>
</tr>
<tr>
<td>(\perp^a)</td>
<td>1.984</td>
<td>310</td>
<td>235</td>
<td>185</td>
<td>170</td>
</tr>
<tr>
<td>(z (|)^a)</td>
<td>1.965</td>
<td>275</td>
<td>275</td>
<td>245</td>
<td>240</td>
</tr>
<tr>
<td>(\text{iso}^b)</td>
<td>1.977</td>
<td>298</td>
<td>248</td>
<td>205</td>
<td>193</td>
</tr>
<tr>
<td>(\text{aniso}^c)</td>
<td>0.019</td>
<td>35</td>
<td>-40</td>
<td>-60</td>
<td>-70</td>
</tr>
<tr>
<td><strong>(T.) elongatus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(\text{Ca}^{2+}) (S_2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x)</td>
<td>1.971</td>
<td>350</td>
<td>249</td>
<td>202</td>
<td>148</td>
</tr>
<tr>
<td>(y)</td>
<td>1.948</td>
<td>310</td>
<td>227</td>
<td>182</td>
<td>162</td>
</tr>
<tr>
<td>(\perp^a)</td>
<td>1.960</td>
<td>330</td>
<td>238</td>
<td>192</td>
<td>155</td>
</tr>
<tr>
<td>(z (|)^a)</td>
<td>1.985</td>
<td>275</td>
<td>278</td>
<td>240</td>
<td>263</td>
</tr>
<tr>
<td>(\text{iso}^b)</td>
<td>1.968</td>
<td>312</td>
<td>251</td>
<td>208</td>
<td>191</td>
</tr>
<tr>
<td>(\text{aniso}^c)</td>
<td>-0.025</td>
<td>55</td>
<td>-40</td>
<td>-48</td>
<td>-108</td>
</tr>
<tr>
<td><strong>(\text{Sr}^{2+}) (S_2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x)</td>
<td>1.995</td>
<td>343</td>
<td>244</td>
<td>200</td>
<td>156</td>
</tr>
<tr>
<td>(y)</td>
<td>1.968</td>
<td>361</td>
<td>217</td>
<td>185</td>
<td>152</td>
</tr>
<tr>
<td>(\perp^a)</td>
<td>1.982</td>
<td>352</td>
<td>231</td>
<td>193</td>
<td>154</td>
</tr>
<tr>
<td>(z (|)^a)</td>
<td>1.957</td>
<td>293</td>
<td>268</td>
<td>223</td>
<td>210</td>
</tr>
<tr>
<td>(\text{iso}^b)</td>
<td>1.973</td>
<td>332</td>
<td>243</td>
<td>203</td>
<td>173</td>
</tr>
<tr>
<td>(\text{aniso}^c)</td>
<td>0.025</td>
<td>59</td>
<td>-37</td>
<td>-30</td>
<td>-56</td>
</tr>
</tbody>
</table>

\(^a\) The equatorial and axial \(G\) and \(A_i\) values are defined as \(G_\perp = (G_x + G_y)/2\), \(G_\parallel = G_z\) and \(A_i,\perp = (A_{i,x} + A_{i,y})/2\), \(A_i,\parallel = A_{i,z}\). \(^b\) The isotropic \(G_{\text{iso}}\) and \(A_{i,\text{iso}}\) \((i = 1\text{-}4)\) values are the averages of the principal values: \(G_{\text{iso}} = (G_x + G_y + G_z)/3\) and \(A_{i,\text{iso}} = (A_{i,x} + A_{i,y} + A_{i,z})/3\). \(^c\) The anisotropy in the \(G\) and \(A_i\) values is expressed as the differences \(G_{\text{aniso}} = G_\perp - G_\parallel\) and \(A_{i,\text{aniso}} = A_{i,\perp} - A_{i,\parallel}\) between the perpendicular and parallel components of the tensors.
Correlation between the ground-to-first excited state energy difference \( \Delta \) and the temperature dependence of the intensity \( I_1 \) of the ground spin state EPR signal. The relative intensity \( I_1 \) of the ground spin state signal \( I_1 \) depends on the inverse temperature weighted by the Boltzmann factor:

\[
I_1 = \frac{C}{T} \frac{(2S_i + 1) \exp(-E_i/kT)}{\sum_i (2S_i + 1) \exp(-E_i/kT)}
\]  

(S1)

where \( C \) is a proportionality constant, \( S_i \) represents the total spin of the coupled states of the spin manifold (\( S = 1/2, 3/2, \ldots \)) and \( E_i \) are the respective energies. Here, a two spin model is used to describe the energy ladder in terms of a single effective coupling constant, \( J_{\text{eff}} \) between two fragments of the Mn tetramer: monomeric Mn\( _{A4} \) (Mn\(^{IV}\), \( S_{A4} = 3/2 \)), and the coupled trimer Mn\( _{B3} \)Mn\( _{C2} \)Mn\( _{D1} \) (Mn\(^{II}\)(Mn\(^{IV}\))\(^2\), spin ground state \( S_{B3-C2-D1} = 1 \) or 2), see (5). The corresponding simplified Hamiltonian \( H = -J_{\text{eff}} S_{A4} S_{B3-C2-D1} \) gives spin state energies \( E_i = (S_{A4}(S_{A4} + 1) + S_{B3-C2-D1}(S_{B3-C2-D1} + 1) - S_i(S_i + 1)) \)

\( J_{\text{eff}} \), where the total spin \( S_i = (S_{A4} - S_{B3-C2-D1}) \ldots (S_{A4} + S_{B3-C2-D1}) = 1/2 \ldots 5/2 \). Equation S1 can thus be used to estimate \( J_{\text{eff}} \) and \( \Delta = E_2 - E_1 = -3J_{\text{eff}} \).
Effect of the Zero-Field Splitting Interaction on the Spin States and EPR and $^{55}$Mn ENDOR Signals of Mn$^{2+}$ Complexes (see also (6)). The EPR and $^{55}$Mn ENDOR signals originating from the Mn$^{2+}$ ions bound to Ca$^{2+}$-depleted PS II differ substantially from those typically associated with mononuclear Mn$^{2+}$ species in that they appear significantly broadened by the large and strongly rhombic ZFS (Figs. 3 and 4A in the main text). The characteristic EPR spectrum of high-spin $S = 5/2$ Mn$^{2+}$ complexes is the six-line signal with a HFI splitting of ~9 mT. For the $^{55}$Mn nucleus of nuclear spin $I = 5/2$, each of these lines can be assigned to one nuclear spin sublevel $m_I$ ranging from -5/2 to +5/2. The corresponding $^{55}$Mn ENDOR signal contains 3 orientation-selective doublets centered roughly around ~125, ~375 and ~625 MHz, which originate from nuclear transitions within the $m_S = |1/2|$, $|3/2|$ and $|5/2|$ electronic submanifolds, respectively.

In the absence of a ZFS interaction, a $^{55}$Mn$^{2+}$ EPR spectrum consist of six separate lines associated with one $m_I$ sublevel, to each of which the five $\Delta m_S = \pm 1$ transitions contribute. The ZFS leads to an anisotropic broadening of these transitions, especially those involving manifolds of electronic spin substates $|m_S| > 1/2$. Additionally, the symmetry of the ZFS tensor has a considerable effect on the orientation-dependence and thus on the line width. A large rhombicity of this interaction enhances the broadening of the powder patterns. As the ZFS becomes more relevant, the transitions associated with a certain $m_I$ are increasingly overlapping, such that it comes to a spreading of the entire spectrum. To some extent, the broadening may additionally be attributed to small site-to-site inhomogeneities of the Mn$^{2+}$ environment, which have an immediate effect on the spin Hamiltonian parameters and are especially found in large and dynamic biological systems like proteins. These effects add up to result in the very broad, featureless spectrum, in which the six-line hyperfine structure from $|m_S| = 1/2$ transitions is not resolved.

The pulse ENDOR spectra are directly affected by this spread. In the absence of a ZFS, the transitions involving a particular $m_I$ can be probed individually by selective irradiation at frequency and field of one of the six EPR lines. Therefore, six different $^{55}$Mn ENDOR spectra can be measured, each of them only comprising the transitions corresponding to the selected nuclear spin $m_I$, associated with all six $m_S$
sublevels. In case of a ZFS-induced anisotropic spread of the $m_S$ substate energies, the relative intensities of the ENDOR lines from the six $m_S$ substates for a particular $m_I$ become orientation-selective. In Fig. 4 (main text), the high-frequency $m_S = -3/2$ signal intensities relative to those of the low-frequency $m_S = -1/2$ and $m_S = +1/2$ signals are clearly smaller at the more central field positions in the EPR spectrum, the spectra at 1208 mT and 1224 mT, compared to the outer ones. At 1195 mT and 1260 mT, there is a stronger relative contribution from the $m_S = -3/2$ transitions due to their larger spread by the ZFS. Furthermore, the overlapping transition energies of the different $m_I$ manifolds result in spectra comprising the ENDOR transitions of more than one $m_I$ value, which leads to a broadening and concomitant lowering of the structural resolution of ENDOR lines. This reduced $m_I$ selectivity can be clearly seen in these very broad $^{55}$Mn ENDOR spectra where no spectral structure of single $m_I$ transitions is resolved. Due to the large energetic spread in the case of the high-spin $m_S = -3/2$ sublevels, even all five corresponding $m_I$ transitions are excited at the same time.
The Electronic Structure of an Effective $S = 1/2$ Spin State System: Relation of Effective Tensor Properties and Non-Explicitly Treated Interaction Terms. For evaluation of the intrinsic ZFS values $d_i$ of the Mn ions resulting from the obtained electronic exchange coupling scheme (Fig. 7 in the main text) and whether these are within the reasonable ranges for the individual Mn oxidation states, a short overview is given on how they are calculated based on the inferred coupling topology and experimental effective spin Hamiltonian parameters:

Same as for the Ca$^{2+}$- and Sr$^{2+}$-containing S$_2$ states, the coupled Mn electronic spin system of the Ca$^{2+}$-depleted S$^*_2$ state can be described by an effective spin Hamiltonian (see the Experimental Procedures section 2.5), which does not include any pair-wise interaction terms such as the Heisenberg-Dirac-Van Vleck operator for the electronic exchange interaction and the ZFS term. Thus, the fitted $G$ and HFI tensors $A_i$ (Table 2 in the main text, supplemental Table S1) also represent effective tensors. However instead, the corresponding intrinsic (on-site) HFI tensors $a_i$ need to be considered for comparison to values reported for other Mn systems, assignment of oxidation states and conclusions about coordination geometries of individual Mn ions. Effective and intrinsic properties of each Mn$_i$ ion are related by a spin projection coefficient $\rho_i$, a measure of the contribution of Mn$_i$ to a particular spin state. The tensor components of this scaling factor are the ratio of the corresponding effective and intrinsic values, i.e. $\rho_i = A_i / a_i$ for the HFI. The effective $G$ tensor, as a property of the effective electron spin $S$ of the Mn cluster, is a weighted linear sum of the intrinsic $g$ tensors of the individual Mn ions $G = \sum \rho_i g_i$. As they map the subspace of the effective spin state to the entire configuration space, the $\rho_i$ tensors for oligomeric spin systems can be computed based on the spin coupling scheme in the form of pair-wise electronic exchange interaction terms between all of the four Mn ions. Therein, the ZFS interaction, not considered explicitly as a term of the spin Hamiltonian, can be taken into account such that it affects the $\rho_i$ tensors in an orientation-dependent manner. Through the exchange coupling between the electronic spins, the intrinsic ZFS value $d_i$ of one Mn ion influences the $\rho_j$ tensors of the others, too, which thus can be envisaged as a transfer of anisotropy to the other Mn ions in the cluster (for more detailed information see Refs. (4, 7, 8)).
REFERENCES
