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

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* Running title: Electronic structure of the Ca$^{2+}$-depleted OEC of Photosystem II

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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.

SUMMARY

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$^2_2$ 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 Mn tetramer. No redistribution of the Mn valence states and only minor perturbation of the exchange interactions between the Mn ions were found. Interestingly, the S$^2_2$ 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 YZ and the Mn 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 $K_D$. Ca$^{2+}$ 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 likely relevant for the formation of the Mn$_4$O$_5$Ca cluster during photoassembly and/or D1 repair.

1. INTRODUCTION

The oxygen-evolving complex (OEC)$^2$ of photosystem II (PS II) catalyzes the light-driven oxidation of water. The OEC contains an inorganic Mn$_4$O$_5$Ca metallocofactor, which 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 YZ (D1-Tyr161) enables electron transfer from the MnA4O5Ca cluster to P680•+, the radical cation formed upon photon absorption and charge separation. The Mn4O5Ca cluster undergoes four successive oxidations, cycling through a series of different net valence states, referred to as the S states (where i = 0–4 denotes the number of oxidizing equivalents stored in the cluster). The transient state S4 spontaneously returns to S0 upon regaining four electrons from the two substrate water molecules, which in the process form molecular oxygen. The release of O2 is followed by the rebinding of at least one H2O molecule, for reviews see refs. (7–14).

X-ray crystallographic structures of the PS II protein complex now provide an atomic picture of the structure of the OEC (1–6), identifying all amino acids that ligate the Mn4O5Ca cluster. The metallocofactor resembles a distorted chair, consisting of the cuboidal moiety Mn4O5Ca (MnB3MnC2MnD13), with the fourth, outer Mn ion (MnA4) connected to the cuboid via an additional μ-oxo bridge (O4) to one of the Mn vertices (MnB3). The reported cluster is likely modified due to photo-reduction of the Mn ions, such that the Mn-Mn and Mn-Ca distances seen in the X-ray structure are all elongated as compared to 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 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 MnA4 connected to MnB3 via a di-μ-oxo bridge (Fig. 1).

The Ca2+ ion of the Mn4O5Ca cluster, which can be removed from and reconstituted into the OEC (19–21), is essential for catalytic function (19–23). The non-catalytic Ca2+-depleted OEC cannot complete the S state cycle, advancing only to a modified S2 state (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 Ca2+ ion during the S state cycle (for reviews see (26–28)). These include:

i. As an integral component of the OEC (6), the Ca2+ ion can be suspected to be of crucial structural importance. However, EXAFS experiments suggest that Ca2+ depletion leads to only a small spatial reorganization of the remnant Mn4O5 cluster (29).

ii. It facilitates fast one electron transfer from YZ•+ to the OEC (for reviews see (11, 30)). The formation of the S2 state requires long visible light illumination at temperatures ≥0°C. This is in contrast to the native S2 state, which can be generated via visible light illumination at -78°C. This apparent increase in the activation energy of OEC turnover upon Ca2+ removal may represent a decoupling of the YZ•+ from the OEC, such that Ca2+-mediated protein conformational changes and/or H+ translocations associated with physiological S state transitions are blocked.

iii. It is a binding/staging site for substrate water and its deprotonation (26, 31). The kinetics of substrate water binding to the OEC are affected by biochemical exchange of Ca2+ with Sr2+, the only surrogate ion able to confer catalytic activity (19, 23, 32). It can presumably act in place of Ca2+ as it has approximately the same size and a similar Lewis acidity (31). This result has been interpreted as evidence for Ca2+ binding one of the substrate waters. Inhibition due to Ca2+ depletion would then reflect the loss of a substrate binding site.

iv. While the basic structural arrangement of Mn ions in the cluster is retained upon Ca2+ removal, it is uncertain if their magnetic and/or electronic interactions are perturbed, which could lead to a decoupling of the cluster or a rearrangement of the Mn valence states. Thus, Ca2+ depletion could potentially change the redox properties, as well as substrate and/or protein interactions of the complex, inhibiting catalytic function.

The Mn4O5Ca cluster in the S2 state exhibits a characteristic multiline EPR signal centered at g ≈ 2 (33), which arises from a 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 a 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 ≈ 4.1 signal (37) (for a full discussion see (41)). The Mn4O5 cluster in the S2 state also exhibits a multiline signal, however, its hyperfine splitting pattern is perturbed. It contains a larger number of resolved lines as compared to the native S2 multiline signal with a smaller average line spacing (5.5-6 mT vs. 8.8 mT). The magnetic interaction between YZ•+ and the OEC is also perturbed in Ca2+-depleted PS II as evidenced...
by changes in the tyrosine split signal of the S2YZ state (24, 25).

A detailed understanding of the electronic structure of the Mn4O5Ca cluster in the S2 state has been developed from pulse EPR data (42–46), in particular 55Mn electron nuclear double resonance (ENDOR). These experiments demonstrated that the four Mn ions contribute about equally to the ground electronic state of the S2 state; i.e., all four Mn ions carry approximately the same spin density. This requirement allows an assessment of the electronic exchange interactions between the four Mn ions and the development of Mn coupling schemes. These necessarily reflect the geometric structure of the OEC and allow the assignment of the individual Mn oxidation states. Our recently proposed model for the S2 state (18) is described in the Discussion section 4.3. This scheme places the only MnIII ion inside the cuboidal unit (MnD1), see also (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 Mn species being able to bind to the Ca2+-depleted PS II has been suggested in an earlier study by Booth et al. (51). The additional species was suggested to be a Mn2+ 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 Ca2+ removal. This was based on the observation that, after equimolar amounts of Mn2+ ions had been added to Ca2+-depleted PS II, no Mn2+ was observed by X-band continuous wave (CW) EPR. Upon titrating Ca2+ ions back into these samples, Mn2+ was released as seen from the appearance of the six-line Mn2+ EPR signal.

In this work, both the spin system of the Mn4O5 cluster in the S2 state of Ca2+-depleted PS II and the binding of Mn2+ ions to this protein were studied by EPR and ENDOR spectroscopy at X- and Q-band frequencies. The results provide new insights into the role of the Ca2+ ion in the native OEC.

2. EXPERIMENTAL PROCEDURES

2.1. 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 N2. Chlorophyll concentrations were determined by assays using aqueous acetone (80 %) extracts (53) with updated extinction coefficients (54) using an ATI Unicam UV/Vis Spectrometer UV2-300.

Ca2+ 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. Ca2+ removal and, as a proof for the integrity of the OEC, Ca2+ rebinding was confirmed both by enzymatic assays and by X-band CW EPR. The O2 evolution rates of native PS II were ~400 μmol O2/mg chlorophyll/h (see the following section). O2 evolution rates dropped to 5-10 % in Ca2+-depleted and were reactivated to >80 % in Ca2+-reconstituted samples. Similar percentages of the S2 multiline signal were observed after white light illumination with a tungsten lamp through an aqueous 5 % CuSO4 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 S1 state EPR samples to the S1 and S1Y2 states (25) was done by illumination at 0 °C for 3 min, with 125 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (10 mM in dimethyl sulfoxide) added to the samples advanced to the S1 state, which restricts the acceptor site, and thus YZ, to one turnover.

For Ca2+ and Mn2+ titration experiments, dark-adapted Ca2+-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 MgCl2, 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 Mn2+ ranging from 0 to 4 equivalents per RC for 2 h. For the Ca2+ titration, samples containing 0.8 added equivalents of Mn2+ were incubated with known amounts of Ca2+ between 0 and 2400 equivalents for one additional hour. Mn2+ and Ca2+ ions were added from stock solutions of their chlorides.

2.2 Oxygen Evolution Measurements. Steady state PS II enzyme activity at 25 °C was determined by polarographic measurement of the O2 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 % CuSO4 IR filter.
The assay medium was the buffer of the samples lacking EDTA and with 5 mM MgCl₂ and 0.2 mM phenyl-β-naphtoquinone (20 mM in dimethyl sulfoxide) added as an electron acceptor.

2.3 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 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 $\pi/2-T-\pi/2-T-\tau$-echo with $\pi = 72$ ns and $\tau = 440$ ns. For $^{55}$Mn Davies ENDOR, the pulse sequence was $\pi-\pi_{RF}-T-\pi-\pi_{RF}-T-\pi/T-\tau$-echo, with $\pi = 12$ ns (X-band), 72 ns (Q-band) or 16 ns (Q-band Mn²⁺ titration/quantification), $\pi_{RF} = 4$ μs (X-, Q-band) or 4.5 μs (Q-band Mn²⁺ titration/quantification), $T = 3.4$ μs (X-, Q-band) or 1 μs (Q-band Mn²⁺ titration/quantification), and $\tau = 200$ ns (X-band), 440 ns (Q-Band) or 320 ns (Q-band Mn²⁺ titration/quantification). The radio frequency (RF) was varied randomly in the desired range and the RF pulses were amplified by an ENI 5100L amplifier. Except for Mn²⁺ titration/quantification, $^{55}$Mn Davies ENDOR spectra were collected using a home-built computer console with SpecMan control software (59) coupled to an SMT02 external RF pulse generator.

2.4 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 Ca²⁺-depleted Mn₄O₅ cluster in the $S^\parallel$ 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:

$$H_{Mn_{4}O_{5},EPR} = \beta_{e}B_{0} \cdot G \cdot S + \sum_{i=1}^{4}(S \cdot A_{i} \cdot I_{i})$$ (1)

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 Mn²⁺ ion ($S = 5/2, I = 5/2$) bound to the Ca²⁺-depleted PS II, the following spin Hamiltonian was solved exactly both for the ESE and ENDOR spectra:

$$H_{Mn^{2+}} = \beta_{e}B_{0} \cdot g \cdot S + D\left(S_{z}^{2} - \frac{1}{3}S(S+1)\right) + E(S_{x}^{2} - S_{y}^{2}) + \beta_{n}B_{0} \cdot g_{n} \cdot I + S \cdot A \cdot I$$ (3)

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

2.5 Temperature-Dependent CW EPR Signal Intensity. The temperature was calibrated using a thermometer in place of the sample in the EPR tube. In order to guarantee that the actual unsaturated intensity $I_{1}$ of the $S^\parallel$ 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 mW 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 b₅₅₉ 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.

2.6 Quantification of the relative concentrations of PS II-bound Mn²⁺ and hexaquo-Mn²⁺. The Mn²⁺ species in Ca²⁺ and Mn²⁺ titration samples were quantified by means of their Q-band $^{55}$Mn Davies ENDOR spectra in two ways and the results were averaged: i) The relative contributions of the spectra from the pure Mn²⁺ species needed to reproduce the spectra from the various titration points were determined. The spectra from Mn²⁺ already present in the Ca²⁺-depleted PS II samples without addition of Mn²⁺ ions and from 40 μM MnCl₂ dissolved in the titration buffer represented PS II-bound and hexaquo-Mn²⁺, respectively. ii) The relative amplitudes of the $^{55}$Mn ENDOR $m_{S} = -3/2$ 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$^{2+}$.

3. RESULTS

3.1 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$_1$ state. It is centered at $g \approx 2$ and spans the magnetic field range from ~260 to ~430 mT, resolving at least 27 hyperfine interaction (HFI) lines with an average peak-to-peak spacing of ~6 mT (Fig. 2A). The central HFI lines are superimposed by the signal of the stable tyrosyl radical Y$_D^*$ centered at $g \approx 2$, which is not depicted for clarity of presentation. The broad underlying signal of the reduced Q$_A^*$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$_1$ 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$_1$ state is essentially invariant across the corresponding EPR signal envelope (supplemental Fig. S1). It is ~130 MHz wide, extending over a range from ~60 to ~190 MHz. As compared to the $^{55}$Mn ENDOR spectrum of the native S$_2$ state (Fig. 2B-c, supplemental Fig. S1), the Ca$^{2+}$-depleted S$_1$ state spectrum is broader. The edges of the spectrum change up to 10 MHz resulting in a ~20 and ~10 MHz increase in the width of the X- and Q-band $^{55}$Mn ENDOR spectra, respectively, as compared to the Ca$^{2+}$-containing S$_2$ state of spinach PS II (42, 45, 46). The Q-band spectrum of the S$_1$ state exhibits five clearly resolved peaks, as also seen for the native S$_2$ state spectrum from T. elongatus (Fig. 2B-d); however, their positions are differing slightly.

The X-band CW EPR and X- and Q-band $^{55}$Mn Davies ENDOR spectra were simultaneously simulated using the spin Hamiltonian formalism (for details see Experimental Procedures section 2.4 and (46, 49)). In these simulations, the S$_1$ state Mn$_4$O$_5$ cluster is treated as an effective $S = 1/2$ electronic spin state coupled to the four $^{55}$Mn nuclei, the same as for the native S$_2$ state (41, 42, 46, 48, 49, 63–66). This approach requires the ground electronic spin state to be well separated from higher states, as is experimentally observed (see the following section). The simulations reproduce all the major spectral features of the EPR and $^{55}$Mn ENDOR spectra (Fig. 2, dashed red traces).

The isotropic and anisotropic values of the fitted effective $^{55}$Mn HFI tensors $A_i$ (i=1-4) are given in Table 2; for means of comparison, the numbers for the native S$_2$ state from spinach (46) and the native and Sr$^{2+}$-substituted S$_2$ states from Thermosynechococcus elongatus (T. elongatus) (49) are also listed. A full set of $G$ and HFI tensor components is listed in supplemental Table S1. As seen for the Mn$_4$O$_5$Ca/Sr clusters, four effective HFI tensors are required to simulate the Mn$_4$O$_5$ cluster spectra. Their magnitudes are of the order seen for mono- and dimeric Mn$^{2+}$ and Mn$^{3+}$ complexes. Hence, their individual spin projection coefficients $\rho_i$ must be on the order of 1 (see ref. (49)). In contrast to preliminary simulations of the S$_1$ spectra (67) or others on the S$_2$ state from spinach PS II (46), the HFI tensors were not constrained to axial symmetry. However, as was found previously in simulations of the Mn$_4$O$_5$Ca/Sr clusters in T. elongatus (49), the tensors nevertheless show a considerable degree of axial symmetry. Moreover, these four OEC clusters show the same geometries of their HFI tensors, with larger axial than equatorial tensor components ($A_{\text{aniso}} < 0$) for $A_1$-$A_4$ and vice versa for the largest HFI $A_1$ ($A_{\text{aniso}} > 0$).

3.2 Spin State Energies of the Ca$^{2+}$-Depleted Mn$_4$O$_5$ Cluster in the S$_1$ State. The energy difference $\Delta$ of the paramagnetic ground spin state and the first exited state was estimated from the temperature dependence of the unsaturated X-band CW modified multiline signal of the Ca$^{2+}$-depleted S$_1$ state. The measured intensities $I_i$ of the derivative signal at a series of temperatures are depicted in a Curie plot vs. $1/T$ in Fig. 3. This relation is approximately linear over the measured range from 14.4 K to 5.5 K and extrapolates to 0 for $T \rightarrow \infty$. This Curie behavior of the temperature dependence indicates that the Ca$^{2+}$-depleted S$_1$ state features an $S = 1/2$ ground spin state energetically well separated from states of higher spin multiplicity. The temperature dependence of the S$_1$ modified multiline signal can be reproduced reasonably well with $\Delta \geq 35$ cm$^{-1}$ corresponding to $J_{\text{eff}} \geq 12$ cm$^{-1}$ (see Experimental Procedures section 2.4). This relatively clear separation from states of higher spin multiplicity allows the S$_1$ 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.

3.3 EPR and $^{55}$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' 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 Ca2+-depleted PS II preparations (S1 state), in which the Mn3O5 cluster does not show a perpendicular mode EPR signal, exhibited a broad EPR signal centered at g = 1.99 with a full width at half maximum of ~63 mT (Fig. 4, inset). A corresponding signal could not be observed with CW X-band EPR spectroscopy; the signal is probably too broad to be discerned from the baseline 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 MHz to 400 MHz (Fig. 4). The 55Mn ENDOR spectra are dominated by two broad peaks between 100-195 MHz and another line centered at ~370 MHz. The two lines at 100-195 MHz are 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 low frequency signals were detected for this species using either ENDOR or electron spin echo envelope modulation (ESEEM).

These EPR and 55Mn ENDOR signals can be readily assigned to high-spin Mn2+ with S = 5/2, although their appearance is different from the spectra typically associated with Mn2+ complexes (see Discussion section 4.1 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 55Mn ENDOR spectra. Besides a near-isotropic G tensor (principal values: 1.983, 1.996, 2.002), the simulations yielded an almost isotropic HFI tensor with the principal components A_x = 256 MHz, A_y = 260 MHz and A_z = 257 MHz resulting in an isotropic average A_iso of 258 MHz. In addition, the simulations required a large fine structure parameter D = -2355 MHz with a pronounced rhombicity η = E/D = -0.38 of the zero-field splitting (ZFS). It is noted that the predominant contribution to the width of the EPR and ENDOR signals is the large and rhombic ZFS interaction (more information on the effect of the ZFS can be found in the Supplemental Data and (61)). Hence, considering the good agreement of the measured and calculated EPR and ENDOR signals (Fig. 4), the optimized fine structure parameter D can be considered robust; i.e., a single set of D and E values is sufficient to rationalize the data. The fact that the inclusion of a distribution of the ZFS parameters is not required indicates that there are only small site-to-site inhomogeneities of the Mn2+ ligand sphere. Therefore, we propose that the Mn2+ ion is bound to one specific site in Ca2+-depleted PS II.

3.4 Mn2+ and Ca2+ Titration Experiments. In order to further investigate the Mn2+ species described in the previous section, Mn2+ and Ca2+ titration experiments of Ca2+-depleted PS II samples were performed, monitoring the CW EPR and ENDOR signal described above.

\( M_{n+}^{2+}/Ca^{2+} \) Titration monitored by CW EPR: Mn2+ ions were added to Ca2+-depleted PS II samples and the characteristic SfY2 state split signal, S1 multoline signal and hexaquo-Mn2+ signal (not shown) were measured. The addition of \( \leq 0.8 \) equivalents of Mn2+ ions relative to the number of PS II RCs did not quantitatively alter all three signals. The Mn2+ ions added are CW EPR-silent, as seen in the study of Booth et al. (51), which is consistent with a protein-bound Mn2+ 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 Mn2+ ions resulted in the appearance of the hexaquo-Mn2+ signal. The subsequent addition of Ca2+ to Ca2+-depleted, Mn2+-loaded PS II samples, led to a loss of the SfY2 state split signal and of the multiline signal, as the Ca2+-reconstituted Mn3O5Ca cluster can proceed beyond the S1 state upon illumination. A concomitant increase of the Mn2+ six-line signal was observed due to the release of the PS II-bound Mn2+ into solution (51).

\( Mn^{2+}/Ca^{2+} \) Titration monitored by 55Mn-ENDOR: Mn2+ binding was also directly monitored by Q-band ENDOR. The concentrations of PS II-bound and solubilized Mn2+ ions in each sample were quantified by means of the relative amplitudes of their characteristic 55Mn ENDOR signals (Fig. 5A, for the titration curve see supplemental Fig. S3). Without the addition of MnCl2, dark-adapted Ca2+-depleted PS II (S1 state) always displayed the PS II-bound Mn2+ signal shown in Fig. 4. The addition of ~0.8 equivalents of MnCl2 led to a 4- to 5-fold increase of this signal with only little free hexaquo-Mn2+ (15 ± 4 %) present at the same time. This suggests that ~20 % of RCs contain a bound Mn2+ before exogenous addition of MnCl2.
so that, in the end, a total of ~1 equivalent 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 ~5% Mn$_4$O$_5$(Ca) clusters. The high occupancy of the Mn 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 range. It is also noted that the addition of Ca$^{2+}$ is expected to be in the submicromolar/nanomolar range. The difference may be due to the Ca$^{2+}$ depletion method used, the low pH/citrate treatment in this study vs. a NaCl salt wash (24) in the study of Booth et al. (51). The difference may be due to the Ca$^{2+}$ depletion method used, the low pH/citrate treatment in this study vs. a NaCl salt wash (24) in the study of Booth et al. (51). Effects on extrinsic PS II subunits could alter the Ca$^{2+}$ binding kinetics, see (24, 51).

4. DISCUSSION

4.1 Location of the Mn$^{2+}$ Binding Site. Based on the observations described above (Results sections 3.3 and 3.4), 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 of the S$_2$Y$_Z$* in 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. It must be at least 10 Å away not to be detectable via dipolar magnetic interaction.

A similar argument holds for Y$_D$* (D2-Tyr160), 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 behaviour 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 T. vulcanus exhibits three additional 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$_D^*$ (Fig. 6A). 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$_{559}$ subunit beta (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 relevance also under physiological conditions. 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 (Figs. 6B, 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), N197 and V198 of the binding motif in Fig. 6B correspond to the conserved residues S286 and V287, while there is no equivalent for T135. E81, E140, H257 in the other binding motif (Fig. 6C) correspond to E146, E205 and K317 (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$^{3+}$, are significantly smaller than those for the PS II-bound Mn$^{3+}$ described here (see Supplemental Data and (61)). The large and highly rhombic ZFS reflects an asymmetric coordination sphere. Both the 7-fold and the 5-fold coordination geometries of the Ca$^{2+}$ ions in PsbO from the two cyanobacterial species exhibit considerable asymmetry (Figs. 6B, C). In addition, the large
proton coupling seen also suggests the Mn$^{2+}$ ion to have at least one water ligand. The absence of any smaller coupling, such as from $^{14}$N, (not shown) indicates that the Mn$^{2+}$ ion does not bind to a N-containing ligand residue like histidine. Thus, the absence of a (visible) water and the presence of His257 as ligands of the Ca$^{2+}$ ion in T. elongatus PsbO (Fig. 6C) favor Mn$^{2+}$ binding to the Ca$^{2+}$ site in PsbO identified in the T. vulcanus crystal structure (Fig. 6B).

PS II from higher plants exhibits an extrinsic subunit composition different from that of the cyanobacterial system. Therein, lumenal PsbP has been reported to be capable of binding Mn$^{2+}$ stoichiometrically (75, 76). Similar to Ca$^{2+}$-depleted PS II in this study and in ref. (51), isolated PsbP loaded with Mn$^{2+}$ did not show a Mn$^{2+}$ X-band CW EPR signal, unless it was denatured. A bound Mn$^{2+}$ could be detected by high field EPR spectroscopy and distinguished from non-specifically attached Mn$^{2+}$, similar to the present study. It is noted though that the binding constant reported in (76) is probably incorrect, for discussion see ref. (77). Moreover, the Mn$^{2+}$ ion in PsbP could be (partially) replaced by Ca$^{2+}$, and Zn$^{2+}$ has been found to bind at one of the two proposed Mn$^{2+}$ sites in PsbP crystals from spinach (PDB # 2VU4) and its cyanobacterial homologue Cyanop (78). Mn$^{2+}$ bound to the PsbP would be at least 30 Å from either the OEC or Y$_D$, again consistent with the distance constraints identified above. Thus, PsbP could also contain the putative site of specific Mn$^{2+}$ binding in Ca$^{2+}$-depleted higher plant PS II.

The physiological role of the putative Mn$^{2+}$ binding site is likely the delivery of Mn to the OEC during photoassembly and/or the storage of Mn$^{2+}$ during the damage/repair cycle of the D1 protein (see (79–82)). Ca$^{2+}$ is essential for photoactivation of the OEC. It was suggested to bind at a site within the PS II complex, which leads to a conformational change of the protein pocket where the OEC is assembled (i.e. the C-terminus of D1). Thus, it appears reasonable that in the absence of Ca$^{2+}$, it is favorable for the PS II supercomplex to sequester in a site Mn$^{2+}$ that can be rapidly delivered upon an increase in Ca$^{2+}$ concentration. In this scenario, the lumenal Ca$^{2+}$ concentration would be a signaling mechanism for OEC assembly and repair.

4.2 Spectral Properties of the Mn$_4$O$_5$ Cluster in the S$_2^-$ State Compared to Other S$_2$ State Systems. The appearance of the $^{55}$Mn ENDOR spectra, the fitted $^{55}$Mn HFI tensors and the ground-to-first excited state energy separation of the Ca$^{2+}$-depleted S$_2^-$ state all fall within the natural spectral variations observed for the native S$_2$ states in different species (41). This demonstrates that the basic electronic and thus also spatial structure of the Mn$_4$O$_5$ cluster remains intact upon Ca$^{2+}$ removal. This confirms and further refines observations on the interatomic distances of the Mn ions from earlier EXAFS experiments (29).

The Ca$^{2+}$-depleted S$_2^-$ state from spinach resembles the native S$_2$ state from T. elongatus with regard to the spin state energies. Upon removal of the Ca$^{2+}$ ion, $\Delta$ increases to $\sim$35 cm$^{-1}$, which is much larger than for the native spinach S$_2$ state ($\Delta = 3-6$ cm$^{-1}$) but more similar to T. elongatus ($\Delta = 12-25$ cm$^{-1}$) (41, 83, 84). In intact spinach PS II, the energy ladder is sensitive to MeOH addition. The mechanism by which MeOH binding perturbs the electronic structure of the S$_2$ state was recently discussed in Su et al. (41). In the model proposed, MeOH binding to the OEC increases, the electronic coupling of the pending Mn (Mn$_{A4}$) to the cuboidal (Mn$_{B3}$,Mn$_{C2}$,Mn$_{D1}$) unit. It is this effective coupling that defines the ground-to-first excited state energy difference $\Delta$ of the S$_2$ state. Ca$^{2+}$ depletion appears to have the same effect. However, addition of MeOH did not modify the appearance of the S$_2^-$ state 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.

4.3 The 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) the ground-to-first excited state energy difference $\Delta$ $\approx$ 35 cm$^{-1}$ (iii) spin projection factors $|\rho\rangle \approx 1$ for all four Mn electronic spins, and (iv) intrinsic ZFS constants $d_i$ of the Mn ions that lie within the range found for mono- and dimeric model complexes, i.e. 1 cm$^{-1}$ $< |d| < 5$ cm$^{-1}$ for Mn$^{III}$ and $|d| < 0.1$ cm$^{-1}$ for Mn$^{IV}$ ions in an octahedral ligand environment (see refs. (18, 47, 49)). The inferred structural (29) and spectral similarity of the native and the Ca$^{2+}$-depleted Mn 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, in which MnB3, MnC2 and MnD1 form a trimeric core unit connected to MnA4 by a di-μ-oxo bridge via MnB3 (17, 18, 47, 85) (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 $J_{A4-C2} = J_{A4-D1} = 0$. The coupling topology fulfills the criteria (i) and (iii), since ground spin state multiplicity and spin projection factors are the same for the two states $S^\uparrow$ and $S^\downarrow$. In contrast, their ground-to-first excited state energy differences $\Delta$ and effective $^{55}$Mn HFI tensors $A_i$, relevant for (ii) and (iv) are different. Thus, the $\Delta = 10.5$ cm$^{-1}$ calculated for the $S^\uparrow$ state coupling scheme also differs from the experimental $\Delta \geq 35$ cm$^{-1}$ determined for the $S^\downarrow$ 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 was shown to be the strength and the sign of the exchange coupling between MnA4 and the trimeric unit comprising MnA4, MnB3 and MnD1. 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, this rationalizes the effect of MeOH on the electronic structure of the MnO$_2$Ca cluster in the native $S^\downarrow$ state (41). For the Ca$^{2+}$-depleted $S^\downarrow$ state, the coupling of MnA4 to the trimeric unit was varied by multiplying the respective exchange coupling constants $J_{A4-B3}$, $J_{A4-C2}$ and $J_{A4-D1}$ 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 $d_i$ of the Mn 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. Due to their inherently small ZFSs, the $d_i$ values of the three Mn$^{III}$ ions can be assumed to be $0$ cm$^{-1}$ for the calculations of the intrinsic HFI tensors $a_i$ from the fitted effective $A_i$ and the computed $\rho_i$ 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 $a_{aniso} = |a|| - |a|$, between the absolute values. Mn$^{IV}$ ions tend to exhibit slightly larger isotropic HFI values ($|a_{iso}| = 187-253$ MHz) and only small intrinsic HFI anisotropies ($|a_{aniso}| < \sim 30$ MHz), see (49). For the Ca$^{2+}$-depleted $S^\downarrow$ state, a ZFS value $d_{D1}$ of the Mn$^{III}_{D1}$ ion in the range of -2.24 to -2.31 cm$^{-1}$ yields $a_i$ tensors consistent with the valence states of the individual Mn ions. An optimized ZFS value $d_{D1} = -2.27$ cm$^{-1}$ leads to the spin projection and intrinsic HFI tensors $\rho_i$ and $a_i$ 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 $d_{D1} = -2.24$ to -2.31 cm$^{-1}$ lies in the range usually found for Mn$^{III}$ ions ($1$ cm$^{-1} < |d| < 5$ cm$^{-1}$), the developed model fulfills the four essential criteria imposed.

### 4.4 Structural Implications of the Zero-Field Splitting $d_{D1}$ of the Mn$^{III}_{D1}$ Ion.

The removal of the Ca$^{2+}$ ion from the spinach OEC is found to results in a significant change of $d_{D1}$ from -1.2 cm$^{-1}$ (41) to -2.2 to -2.3 cm$^{-1}$, larger than for the Ca$^{2+}$/Sr$^{2+}$ exchange in PS II from $T$. elongatus. For these systems, the intrinsic ZFS values of the Mn$^{III}_{D1}$ ion are relatively similar (Ca$^{2+}$: $d_{D1} = -1.3$ cm$^{-1}$, Sr$^{2+}$: $d_{D1} = -1.2$ cm$^{-1}$) (49). It is however noted that the signs of the $d_{D1}$ and of the HFI anisotropy of the Mn$^{III}$ ion do not change between the Ca$^{2+}$-depleted $S^\downarrow$ 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 $^5B_{1g}$ 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$^{III}_{D1}$ for the $S^\downarrow$ 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 μ-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 (18), could be reoriented upon Ca$^{2+}$ depletion leading to a distortion of the coordination sphere and thus alter $d_{D1}$.

In the latest crystal structure, all four Mn ions are 6-coordinate (6). This however requires the O5 μ-oxo bridge to be a ligand of MnA4, MnB3 and
MnD1, engendering very long Mn-O5 bond distances, well outside the range seen in model complexes (see (18)) and by EXAFS spectroscopy of the Mn4O5Ca 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 towards the MnA4, forming a genuine μ-oxo bridge between MnA4 and MnB3 and results in MnD1 having an open coordination site. In this case, in the Ca2+-depleted S2 state, Glu189 might function as a bidentate ligand in a then tetragonally elongated 6-coordinate MnD1III ligand sphere, leading to the observed change of dD1.

Alternatively, the absence of Ca2+ may allow this open site to be occupied by a water molecule in the S2 state (Fig. 8) forming a sixth ligand to MnD1. The MnD1-bound water molecule is the second substrate water in the mechanism proposed by Siegbahn (17), which potentially binds during the S2- to-S3 transition. Thus, within this model, one of the roles of Ca2+ 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) but lead to single product (O2) formation. Consistent with this role for the Ca2+ ion is the known S state dependence of the affinity of Ca2+ to this site (93). It drops significantly in the S2 state, suggesting that in this state, Ca2+ is less tightly bound, having a more flexible ligand sphere that potentially allows greater solvent access to the MnD1 ion.

Besides μ-oxo bridge protonation, the loss of two positive charges is likely to be compensated by protonation of amino acid residues ligating the Ca2+ ion in the intact cluster. Other possibilities are the replacement of Ca2+ by monovalent Na+ in the samples or the absence of complete charge compensation, leaving the Mn4O5 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 (PCET) to YZ. In the light of the proposed deprotonation scheme 1,0,1,2 for the individual oxidation steps starting from S0 (94), this would explain the Mn4O5 cluster being able to advance to S3 but not from S2YZ to S3.

5. CONCLUSIONS

The present study demonstrates that Ca2+ is not required for conferring the critical electronic properties to the Mn4O5Ca cluster. This also confirms that Ca2+ is not essential for structural maintenance of the OEC. Its presence or absence does not affect the position of the only MnIII ion of the cluster in the S2/S2 state (MnD1), and the contribution of the four Mn ions to the electronic states S2 and S2 does not differ considerably. Thus, the necessity for Ca2+ in water splitting catalysis must be due to another functional role of the Ca2+ ion.

While the exact mechanism of inhibition upon Ca2+ 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 Ca2+-bound and a Mn-bound substrate water (be it a terminal ligand MnV=O or a μ-oxo bridge) (11, 95–97), inhibition due to Ca2+ 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 S2YZ*. (ii) Instead, O-O bond formation has been proposed to follow a mechanism that results in the coupling of substrates bound to two Mn sites (be it between two terminal bound Mn-O ligands or involving a μ-oxo bridge via oxyl radical coupling) (10, 14, 16, 17, 98). Then, inhibition due to Ca2+ removal probably represents a secondary effect, where the Ca2+ ion is critical for maintaining the H-bond network between YZ and the Mn cluster (6, 11, 30), as opposed (or in addition) to perturbation of substrate binding. Thus, Ca2+ removal would disable PCET during the S2YZ* to S3 transition, preventing substrate deprotonation and concomitant oxidation of MnD1III. Thus, the elucidation of the mechanistic role of the Ca2+ ion in the OEC is tightly linked to understanding the mechanism of photosynthetic water splitting.

REFERENCES


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FOOTNOTES

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2The abbreviations used are: OEC, oxygen-evolving complex; PS II, photosystem II; EXAFS, extended X-ray absorption fine structure; MeOH, methanol; ENDOR, electron-nuclear double resonance; CW, continuous wave; RC, reaction center; ESE, electron spin echo; RF, radio frequency; HFI, hyperfine interaction; T. elongatus / T.e., Thermosynechococcus elongatus; ZFS, zero-field splitting; DFT, density functional theory.

3The nomenclature used for the Mn ions combines the lettering/numbering used in polarized EXAFS models (90) and that of Umena et al. (6).
FIGURE 1. Stereo view of a DFT model of the Mn₄O₅Ca cluster in the S₂ state and directly ligating amino acid residues and H₂O/OH- molecules. (18). Amino acids, except CP43-Glu354, are from PS II subunit D1. Mn, Ca, N, O, C, and H atoms are shown in purple, green, blue, red, gray, and white, respectively. Nonpolar H atoms are omitted for clarity.

FIGURE 2. EPR and ENDOR experimental spectra (black solid traces) and simulations (red dashed traces). (A) X-band CW EPR of PS II isolated from spinach. (a) Ca²⁺-depleted OEC poised in the S₂⁺ state, (b) native, (c) Ca²⁺-depleted and (d) Ca²⁺-reconstituted OECs illuminated at 200 K. In the experimental spectrum, the region of the overlapping YD⁺ signal (g ≈ 2) was omitted for clarity. In (a), a 4th order polynomial, in (b-d), a background signal of the resonator cavity, were subtracted from the raw data. Experimental parameters: MW frequencies: 9.634 GHz (a), 9.44 GHz (b-d); MW power: 0.5 mW (a), 20 mW (b-d); modulation amplitude: 7.5 G (a), 15 G (b-d); time constant: 82 ms; temperature: 8 K (a), 10 K (b-d). (B) X-band (a) and Q-band (b-e) Davies ENDOR of the Ca²⁺-depleted S₂⁺ state from spinach, compared with the native and Sr²⁺-substituted S₂ states from spinach and Thermosynechococcus elongatus (T.e.). (a), (b) Ca²⁺-depleted Mn₄O₅ S₂⁺ spinach, (c) native Mn₄O₅Ca S₂⁺ spinach, (c) native Mn₄O₅Ca S₂⁺ (taken from (45, 46)), (c) native Mn₄O₅Ca S₂⁺, T.e. (from (49)), (c) Sr²⁺-substituted Mn₄O₅Sr S₂⁺, T.e. (from (49)). (a) and (b) were smoothed using a 9- and 5-point moving average, respectively. (b) is the difference of an S₂⁺ state spectrum after illumination at 0 °C minus an S₁⁺ state spectrum after dark-adaptation (supplemental Fig. S2) in order to remove an overlapping Mn²⁺ signal. The Mn²⁺ signal is attributed to residual Mn²⁺ ions stemming from a small fraction of damaged Mn clusters. In X-band pulse EPR (not shown) and ENDOR spectra (a), Mn²⁺ contributions were avoided by optimizing the MW pulse lengths for the S₂⁺ state signal of the Mn₄O₅ spin system with an S = 1/2 ground spin state. Experimental parameters: (a, b) MW frequencies: 9.717 GHz (X-band), 34.033 GHz (Q-band); shot repetition rate: 5 μs; MW pulse lengths π: 12 ns (X-band), 72 ns (Q-band); τ: 200 ns (X-band), 440 ns (Q-band); magnetic fields: 380 mT (X-band), 1208 mT (Q-band); RF pulse length πRF: 4 μs; temperature: 5 K. (c-e) see (45, 49).

FIGURE 3. Curie plot showing the dependence of the intensity I₁ of the modified multiline derivative signal of the Ca²⁺-depleted S₂⁺ state on the inverse temperature T. The error of the x-values comes from the calibration of the actual temperature at the sample position (see Experimental Procedures section 2.4). The curves are simulations of the Curie temperature dependence over a range of Δ values on the basis of Equation S1 in the Supplemental Data and the simplified electron 2-spin coupling scheme for the OEC outlined in the Experimental Procedures section 2.5. Experimental parameters: MW frequency: 9.437 GHz; MW power: 0.1 mW; modulation amplitude: 7.5 G; time constant: 82 ms; temperatures: 5.5 K, 6.3 K, 7.3 K, 8.7 K, 14.4 K.

FIGURE 4. Q-band pulse ESE-detected field-swept EPR (inset) and Davies ENDOR experimental spectra (black solid traces) and simulations (red dashed traces) of the Mn²⁺ ion bound to Ca²⁺-depleted PS II isolated from spinach and poised in the S₁⁺ state. In the EPR spectrum (inset), the region of the overlapping YD⁺ EPR signal (g ≈ 2) is not displayed for clarity and was omitted in the simulations. The arrows indicate the four magnetic fields at which the ENDOR spectra were measured. Experimental parameters: MW frequency: 34.07 GHz; shot repetition rate: 5 μs; MW pulse length π: 72 ns; τ: 440 ns; Davies ENDOR: magnetic fields: 1195 mT, 1208 mT, 1224 mT, 1260 mT (top to bottom); RF pulse length πRF: 4 μs; temperature: 5 K.

FIGURE 5. (A) Q-band ⁵⁵Mn Davies ENDOR spectra of dark-adapted Ca²⁺-depleted PS II samples (S₁⁺ state) with 0.16, 1.2 and 4.0 (black, red and blue trace) equivalents of Mn²⁺ ions added, relative to the number of PS II RCs, and of 40 μM MnCl₂ (corresponding to 1.6 equivalents) dissolved in the same buffer used for the PS II titration experiments. For the titration curve see supplemental Fig. S3. The spectra were smoothed using a 5-point moving average. (B) Titration of Ca²⁺-depleted PS II samples containing 1 equivalent of Mn²⁺ ions with respect to the PS II RCs with Ca²⁺. The relative ⁵⁵Mn ENDOR signal amplitudes of Mn²⁺ ions bound to the PS II protein complex (black squares) and hexaquo-Mn²⁺ in solution (red circles), quantified as described in the Experimental Procedures section 2.6, are plotted against the equivalents of Ca²⁺ ions added to the samples. The concentrations of both Mn²⁺ species as a function of added Ca²⁺ were reproduced by a sigmoid fit curve (solid lines). The concentration of RCs in the samples was 25 ± 3 μM. Experimental parameters: MW frequency: 34.03 GHz; shot repetition rate: 5 μs; MW pulse length π: 200 ns; modulation amplitude: 7.5 G; time constant: 82 ms; temperature: 5 K.
μs; MW pulse length $\pi$: 16 ns; $\tau$: 320 ns; magnetic field: 1224 mT; RF pulse length $\pi_{RF}$: 4.5 μs; temperature: 5 K.

**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 # 3ARC) highlighting the Ca$^{2+}$ ions (black spheres), as well as a Ca$^{2+}$ binding site found in PS II from *T. elongatus* (grey sphere), their distances to the paramagnetic entities Mn$_4$O$_5$Ca cluster, Y$_Z^*$ and Y$_D^*$. 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), (C) Ligand environments of the Ca$^{2+}$ ions in the extrinsic PsbO proteins from *T. vulcanus* and *T. elongatus* (5), respectively. O, N and C atoms are shown in red, blue and yellow, respectively. H atoms are omitted for clarity. 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 7.** Model for the electronic structure of the OEC in the native S$_2$ and Ca$^{2+}$-depleted S$_2'$ states calculated based on a refined DFT structure of the OEC (18) in the latest crystal structure (6). A-D label the Mn ions in their respective oxidation state, the numbers give the pair-wise exchange coupling $J_{ij}$ between the electronic spins of the Mn ions in cm$^{-1}$. The constant $c$ is 1 in the originally derived model, but differs for the various clusters and conditions, such as the presence or absence of MeOH. The S$_2'$ state can be described by the scheme with $c = 1.65$.

**FIGURE 8.** Scheme of the native Mn$_4$O$_5$Ca cluster in the S$_2$ state and the Ca$^{2+}$-depleted S$_2'$ state represented by a hypothesized Mn$_4$O$_5$ cluster. There, the fast exchanging substrate water is already in the S$_2'$ state bound to Mn$_{D_1}^{III}$ filling the space of the Ca$^{2+}$ ion. W$_s$ and W$_f$ denote the slowly and fast exchanging substrate waters, respectively (96, 99).
the S2' State (Fig. 2) and for the S2 States of Native Spinach PS II (46) and Native and Sr2+-substituted PS II from Spinach in the Ca2+-depleted S2' state on the Basis of the Electronic Exchange Coupling Scheme. The anisotropy in the equatorial and axial components of the tensor. The equatorial and axial \( A_i \) values are defined as: 
\[
A_{i,\perp} = (A_{i,x} + A_{i,y})/2, \quad A_{i,\parallel} = A_{i,z}.
\]

### TABLE 1. Oxygen Evolution Activities and Relative S2 Multiline EPR Signal Intensities of the Ca2+-containing Native, the Ca2+-depleted and Ca2+-reconstituted PS II Membrane Preparations from Spinach.

<table>
<thead>
<tr>
<th></th>
<th>Native</th>
<th>Ca2+-depleted</th>
<th>Ca2+-reconstituted</th>
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<tbody>
<tr>
<td>Enzymatic rates / ( \mu \text{mol O}_2/\text{mg chlorophyll/h} )</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 S2 state multiline signal intensities</td>
<td>100 %</td>
<td>8 ± 3 %</td>
<td>105 ± 12 %</td>
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* Determined as an average of at least 8 single measurements at a minimum of 2 different chlorophyll concentrations from 5 - 25 \( \mu \text{g/ml} \).  
* Determined from the peak-to-trough distances of 4 prominent derivative peaks in the CW EPR spectrum (100).

### TABLE 2. Isotropic and Anisotropic Values of the Effective 55Mn HFI Tensors \( A_i \) (\( i = 1-4 \)) for the Simulations of the X- and Q-Band EPR and ENDOR Spectra of the Ca2+-depleted PS II from Spinach in the S2 State (Fig. 2) and for the S2 States of Native Spinach PS II (46) and Native and Sr2+-substituted PS II from \( T. \ elongatus \) (49).

<table>
<thead>
<tr>
<th></th>
<th>( A_1 \text{ / MHz} )</th>
<th>( A_2 \text{ / MHz} )</th>
<th>( A_3 \text{ / MHz} )</th>
<th>( A_4 \text{ / MHz} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach -Ca2+ S2</td>
<td>311</td>
<td>234</td>
<td>202</td>
<td>171</td>
</tr>
<tr>
<td>( \text{iso} )</td>
<td>72</td>
<td>-84</td>
<td>-38</td>
<td>-59</td>
</tr>
<tr>
<td>( \text{aniso} )</td>
<td>35</td>
<td>-40</td>
<td>-60</td>
<td>-70</td>
</tr>
<tr>
<td>Ca2+ S2</td>
<td>312</td>
<td>251</td>
<td>208</td>
<td>191</td>
</tr>
<tr>
<td>( \text{iso} )</td>
<td>55</td>
<td>-40</td>
<td>-48</td>
<td>-108</td>
</tr>
<tr>
<td>( \text{aniso} )</td>
<td>59</td>
<td>-37</td>
<td>-30</td>
<td>-56</td>
</tr>
<tr>
<td>( T. \ elongatus ) Ca2+ S2</td>
<td>332</td>
<td>243</td>
<td>203</td>
<td>173</td>
</tr>
<tr>
<td>( \text{iso} )</td>
<td>59</td>
<td>-37</td>
<td>-30</td>
<td>-56</td>
</tr>
<tr>
<td>( \text{aniso} )</td>
<td>332</td>
<td>243</td>
<td>203</td>
<td>173</td>
</tr>
</tbody>
</table>

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

### TABLE 3. Calculated Spin Projection Tensor Components \( \rho_\parallel \) and \( \rho_\perp \), Intrinsic 55Mn HFI Tensor Components \( a_i \) and \( a_i' \), and Isotropic and Anisotropic Intrinsic HFI Values \( a_{\text{iso}} \) and \( a_{\text{aniso}} \) for the Mn Ions of the OEC in the Ca2+-depleted S2' state on the Basis of the Electronic Exchange Coupling Scheme in Fig. 7 with \( c = 1.65 \) and Intrinsic ZFS values \( d_{A4} = d_{B3} = d_{C2} = 0 \text{ cm}^{-1} \) for the MnIV ions and \( d_{D1} = -2.27 \text{ cm}^{-1} \) for the MnD1III ion.

<table>
<thead>
<tr>
<th></th>
<th>( \rho_\parallel )</th>
<th>( \rho_\perp )</th>
<th>( a_{i, \text{MHz}} )</th>
<th>( a_{i', \text{MHz}} )</th>
<th>( a_{\text{iso}, \text{MHz}} )</th>
<th>( a_{\text{aniso}, \text{MHz}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{MnA4 (MnIV)} )</td>
<td>1.03</td>
<td>1.25</td>
<td>197</td>
<td>230</td>
<td>208</td>
<td>33</td>
</tr>
<tr>
<td>( \text{MnB3 (MnIV)} )</td>
<td>-0.81</td>
<td>-1.09</td>
<td>187</td>
<td>190</td>
<td>188</td>
<td>3</td>
</tr>
<tr>
<td>( \text{MnC2 (MnIV)} )</td>
<td>-0.87</td>
<td>-1.21</td>
<td>220</td>
<td>188</td>
<td>209</td>
<td>-31</td>
</tr>
<tr>
<td>( \text{MnD1 (MnIII)} )</td>
<td>1.66</td>
<td>2.04</td>
<td>202</td>
<td>123</td>
<td>175</td>
<td>-79</td>
</tr>
</tbody>
</table>

* The equatorial and axial \( a_i \) values are defined as: \( a_\perp = (a_x + a_z)/2 \), \( a_\parallel = a_y \).  
* The isotropic \( a_{\text{iso}} \) values are the averages of the individual components of the tensor \( a_{\text{iso}} = (a_x + a_y + a_z)/3 \).  
* The anisotropy of the \( a \) tensor is expressed as the difference \( a_{\text{aniso}} = a_\parallel - a_\perp \) between the parallel and perpendicular tensor components.
Figure 3

Data

Simulations:
- $\Delta = 6$ cm$^{-1}$
- $\Delta = 20$ cm$^{-1}$
- $\Delta = 35$ cm$^{-1}$
- $\Delta = 60$ cm$^{-1}$

$I_1$ / a.u.

$T^{-1}$ / K$^{-1}$

0.00 0.05 0.10 0.15 0.20
Figure 5

(A) ENDOR Amplitude / a.u. vs. Radio Frequency / MHz for Ca\(^{2+}\)-depleted PS II with different Mn\(^{2+}\) concentrations:
- Black line: +0.16 eq. Mn\(^{2+}\)
- Red line: +1.2 eq. Mn\(^{2+}\)
- Blue line: +4.0 eq. Mn\(^{2+}\)
- Cyan line: 1.6 eq. Mn\(^{2+}\)

(B) Relative Mn\(^{2+}\) ENDOR Signal Amplitudes vs. Added Ca\(^{2+}\) per PS II Reaction Center:
- Black square: Mn\(^{2+}\)-PS II
- Red circle: Mn\(^{2+}\) x 6 H\(_2\)O
- Black line: Sigmoid Fits
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 and Wolfgang Lubitz

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