On the structure of the manganese complex of photosystem II: extended-range EXAFS data and specific atomic-resolution models for four S-states

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The water-oxidizing manganese complex bound to the proteins of photosystem II (PSII) was studied by X-ray absorption spectroscopy on PSII membrane particles. An extended range for collection of extended X-ray absorption fine-structure (EXAFS) data was used (up to 16.6 Å−1). The EXAFS suggests the presence of two Mn–Mn distances close to 2.7 Å (per Mn4Ca complex); the existence of a third Mn–Mn distance below 2.9 Å is at least uncertain. Interestingly, a distance of 3.7 Å is clearly resolved in the extended-range data and tentatively assigned to a Mn–Mn distance. Taking into account the above EXAFS results (inter alia), we present a model for the structure of the PSII manganese complex, which differs from previous atomic-resolution models. Emphasizing the hypothetical character, we propose for all semi-stable S-states: (i) a structure of the Mn4Ca(μ-O)ₙ core, (ii) a model of the amino acid environment, and (iii) assignments of distinct Mn oxidation states to all the individual Mn ions. This specific working model may permit discussion, verification and invalidation of its various features in comparison with experimental and theoretical findings.

Keywords: photosynthetic oxygen evolution; photosynthesis; water oxidation; X-ray absorption spectroscopy

Abbreviations: EXAFS, extended X-ray absorption fine-structure; FT, Fourier transform; PSII, photosystem II; FTIR, Fourier-transform infrared (spectroscopy); YZ, Tyr160/161 of the D1 protein; XANES, X-ray absorption near-edge structure; XAS, X-ray absorption spectroscopy

1. INTRODUCTION

Driven by the absorption of four photons, plants and cyanobacteria oxidize water, thereby producing atmospheric dioxygen (McEvoy & Brudvig 2006). Water oxidation by photosystem II (PSII) is of fundamental importance for the atmosphere (O₂ production) and biosphere (primary biomass formation). Understanding its mechanism could promote the development of biotechnological and biomimetic approaches for light-driven H₂ production from water.

The catalyst of water splitting is a pentanuclear Mn₄Ca complex (denoted here as Mn complex) bound to the proteins of PSII. The crystallographic model of Zouni et al. (2001) of PSII revealed the position of the Mn complex with respect to other redox factors. Barber and his co-workers provided a specific hypothesis on the structure of the Mn complex and its ligand environment and showed that the Mn complex includes a Ca ion and thus is a pentanuclear Mn₄Ca complex (Ferreira et al. 2004), thereby confirming earlier extended X-ray absorption fine-structure (EXAFS) results (Cinco et al. 2002). At an improved resolution of 3.0 Å, Loll et al. (2005) assigned residues ligating the metal ions of the Mn complex differently.

A definitive structural model of the Mn₄Ca complex cannot be concluded from the available crystallographic data alone, inter alia owing to Mn reduction by X-ray exposure during data collection (Dau et al. 2004; Loll et al. 2005; Yano et al. 2005a; Grabolle et al. 2006). Results obtained by EXAFS spectroscopy have been incorporated in all crystallographic models and in general may be useful in construction of structural models of the Mn complex in its protein environment (Yano et al. 2006). In the present work, we present new extended-range EXAFS data and a specific working model on the structure of the Mn complex in the four semi-stable S-states, which represent the experimentally easily accessible subset of the nine (or more) functional intermediates of the reaction cycle of photosynthetic dioxygen formation as reviewed elsewhere (Dau & Haumann 2006, 2007, 2008).

2. MATERIAL AND METHODS

Highly active PSII membrane particles were prepared from spinach (Schiller & Dau 2000). Preparation of XAS samples (approx. 300 μm layers of PSII particles), XAS data collection (bending-magnet beamline, EMBL outstation Hamburg at the DESY) and data analysis procedures are described elsewhere (Iuzzolino et al. 1998; Schiller et al. 1998; Haumann et al. 2005). The X-ray energy was scanned from 6420 to 7610 eV (sample temperature of 20 K; ‘magic’ excitation angle of approx. 55° (Dittmer & Dau 1998); single scan per irradiated spot; approx. 75 scans of approx. 40 min
An energy-resolving 13-element Ge detector (Canberra) was employed; in 11 discriminators the windows were set for detection of the Mn $K_a$-fluorescence and in two for the detection of the Fe fluorescence. After subtraction of the Fe contribution (see further below), normalization and extraction of the EXAFS oscillations, the energy scale of the EXAFS spectrum was transformed to a $k$-scale employing an $E_{0}$ of 6540 eV. For calculation of the shown Fourier transforms, $\cos^2$-window functions extending at the low $k$-side over 10% and at the high $k$-side over 20% of the respective $k$-range were used.

The $k^3$-weighted EXAFS spectra were simulated for $k$-values ranging from 1.2 to 16.6 Å$^{-1}$ (phase-function calculation by the PEPP program (Rehr et al. 1991); $S_0^2 = 0.85; E_{0} = 6547$ eV). The use of Fourier-isolation techniques was strictly avoided. Only subsequent to the curve-fit itself, the quality of the least-square fits was judged by calculation of the Fourier-filtered $R$-factor ($R_D$) described by Meinke et al. (2000).

For structural modelling, the potential energy was minimized using a classical force field (MM+ force field of HyperChem 7, HyperCube). Since the molecular-mechanics force field cannot model the geometry of the metal complex itself, it was supplemented by a set of restraints involving all Mn ligand (first-sphere ligands only) and all intermetal distances (shorter than 4 Å) as well as selected bond angles.

### 3. RESULTS AND DISCUSSION

#### (a) Extended-range EXAFS

To extend the EXAFS range beyond the Fe K-edge, we used the procedure described by Gu et al. (2003). The $K_a$-fluorescence predominantly resulting from Mn excitation (detection at approx. 5899 eV) and the $K_b$-fluorescence from iron (6404 eV) were simultaneously recorded. In the Mn signal, the iron-edge at approximately 7110 eV is clearly visible (figure 1, dotted line). By a weighted subtraction of the simultaneously measured Fe signal (dashed line), the Fe contribution to the Mn signal was completely removed, as visible in the inset of figure 1. The thereby obtained EXAFS spectrum (solid line) extends up to $k$-values of 16.6 Å$^{-1}$ (figure 2a).

The benefit of the extended range becomes apparent by comparison of the Fourier-transformed spectra (figure 2b). As anticipated, the FT peaks assignable to Mn–Mn/Ca vectors become enhanced. Peak II is dominated by contributions from Mn–Mn vectors of 2.7 Å length; it narrows and increases pronouncedly in magnitude. Peak III at a reduced distance of approximately 3 Å splits into one peak at 2.85 Å (III-a) and a second peak at 3.3 Å (III-b), suggesting the presence of backscattering atoms at approximately 3.3 Å as well as at 3.7 Å. In previous extended-range EXAFS studies, the peak III-a also shifted to shorter distances in comparison with the conventional-range spectrum, but the peak III-b remained below the noise level (Yano et al. 2005b; Pushkar et al. 2007).

Since Fourier-isolation techniques are potentially misleading (Dau et al. 2004), the original $k^3$-weighted EXAFS spectrum was simulated. The peak III-b is reproduced only if backscattering atoms at 3.7 Å distance are taken into account (table 1). In an early study, the 3.7 Å EXAFS was assigned to a Ca ion at approximately 3.7 Å (MacLachlan et al. 1992). In past studies of our group, several light atoms at a distance of 3.7 Å to manganese were employed in the EXAFS simulations.
Extended-range EXAFS and structural model

H. Dau et al.

Table 1. Parameters obtained by simulation of the $k^3$-weighted EXAFS spectrum of figure 2. (The respective pair of the X-ray absorbing (A=Mn) and electron-backscattering atom (B=Mn, Ca, O) is indicated in the first row. For each backscatterer shell, the simulation parameters are given, i.e. the A-B distance ($R_{AB}$, first line in each box), the Debye-Waller parameter ($\sigma$, second line), the number of backscattering atoms per Mn ion ($N_B$, third line; the corresponding number of distance vectors per Mn$_4$ complex is given in parentheses). Numbers which had been fixed in the fit of the unfiltered $k^3$-weighted EXAFS oscillations are marked by *. The first-sphere ligands were simulated using two shells of oxygen atoms resulting in the following parameters (for all simulations in the table): 1.85 Å, 2.06 Å ($R_1$, $R_2$); 0.07 Å, 0.13 Å ($\sigma_1$, $\sigma_2$); 3, 2.75* ($N_1$, $N_2$). In the last row, the EXAFS $R$-factor providing the deviation between simulated and experimental spectra has been calculated for the EXAFS oscillations that correspond to reduced distances between 2.2 and 3.6 Å (peaks II, III-a and III-b; Fourier-filtered $R$-factor). The quality of the simulations d, e and f is essentially identical, and f is disfavoured only because the presence of a high number of Mn–O vectors of 3.6 Å length is highly unlikely.)

<table>
<thead>
<tr>
<th>Mn–Mn</th>
<th>Mn–Mn</th>
<th>Mn–Ca</th>
<th>Mn–Mn [Mn–O]</th>
<th>$R_t$ (%)</th>
</tr>
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<tbody>
<tr>
<td>(a)</td>
<td>2.72 Å</td>
<td>0.04 Å</td>
<td>1.10</td>
<td>27</td>
</tr>
<tr>
<td>(b)</td>
<td>2.72 Å</td>
<td>3.28 Å</td>
<td>0.03 Å</td>
<td>0.06 Å</td>
</tr>
<tr>
<td>(c)</td>
<td>2.72 Å</td>
<td>3.28 Å</td>
<td>3.71 Å</td>
<td>11</td>
</tr>
<tr>
<td>(d)</td>
<td>2.72 Å</td>
<td>3.25 Å</td>
<td>3.29 Å</td>
<td>6</td>
</tr>
<tr>
<td>(e)</td>
<td>2.72 Å</td>
<td>3.27 Å</td>
<td>3.36 Å</td>
<td>7.1 Å</td>
</tr>
<tr>
<td>(f)</td>
<td>2.72 Å</td>
<td>3.29 Å</td>
<td>3.44 Å [3.63 Å]</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>0.03 Å</td>
<td>0.06 Å</td>
<td>0.07 Å [0.06 Å]</td>
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<tr>
<td></td>
<td>1.02 (2)</td>
<td>1.0* (2)</td>
<td>0.5* (2)</td>
<td>[2.54] (10)</td>
</tr>
</tbody>
</table>

(Mn or Ca). Acceptable simulation results are also obtained for light backscatterers, but only for a larger number of these (5–20 Mn–O/N vectors per Mn complex) within a narrow distance range (small $\sigma$-value). Since all more recent structural models of the PSII manganese complex are essentially incompatible with a larger number of Mn–O vectors close to 3.7 Å, we consider the assignment to light atoms as less likely. No indications of 3.7 Å Mn–Ca vectors have been found in EXAFS studies at the Ca or Sr K-edge (Cinco et al. 2002, 2004; Müller et al. 2005). Thus, we tentatively assign the 3.7 Å distance to a Mn–Mn vector.

For the extended-range data, the coordination number is close to unity for the 2.7 Å EXAFS interaction suggesting the presence of two Mn–Mn vectors of approximately 2.7 Å length (table 1). For fits involving a further Mn–Mn distance in the range from 2.77 to 2.97 Å, the coordination number of this additional shell consistently drops below 0.15 (mostly below 0.08) suggesting that the data are incompatible with the presence of an additional Mn–Mn vector of 2.8–2.9 Å length. Possible reasons for the divergent conclusion of Yano et al. (2005b) and Pushkar et al. (2007) have been discussed by Dau et al. (2004).

We conclude: (i) a Mn–Mn distance close to 3.7 Å may be present in the Mn complex of PSII; (ii) the EXAFS fits point towards the existence of not more than two Mn–Mn vectors of less than 2.9 Å length (per Mn$_4$Ca complex), the existence of a third one being at least uncertain. In the following, we will present a working model for the structure of the PSII manganese complex for various S-states which inter alia takes into account the above points and represents an alternative to recently proposed structural models (Ferreira et al. 2004; Yano et al. 2006).

(b) Structural model of the Mn complex

(i) S$_1$-state complex

The ligation of the metal ions by amino acid residues was modelled according to Loll et al. (2005). The $\mu$-oxo bridging between metal ions was chosen such that the final model matches the EXAFS results which we have obtained on isotropic and unidirectionally oriented PSII samples, and can account in a straightforward way for the structural changes in the S$_1$-state cycle suggested elsewhere (Dau et al. 2001, 2003; Haumann et al. 2005). Molecular mechanics modelling was employed where a standard force field had been complemented by chemically reasonable restraints regarding the Mn and Ca ligand environment; the restraints for the intermetal distances were chosen in accord with the EXAFS results (table 1e). Starting with the coordinates of Loll et al. (2005; figure 3), minimization of the potential energy was carried out by allowing for changes in the coordinates of atoms within a narrow range around Mn$_1$, Mn$_2$ and Mn$_3$, but within a clearly extended range around Mn$_4$, to account for the putative influence of radiation-induced modifications on Mn$_4$ and its ligand environment suggested by the analysis in Loll et al. (2005). In the thereby obtained structure (figures 3 and 4), Mn$_1$ and Mn$_2$ are close to the original coordinates of Loll et al. (2005), Mn$_3$ is moderately

Figure 3. Overlay of initial and final structure in molecular mechanics modelling of the protein environment of the Mn complex. Energy minimization leads from the starting structure (light grey bonds and atoms, Mn$_3$ and Mn$_4$ labelled as 3 and 4, respectively) to the here discussed atomic-resolution model of the Mn complex and its ligand environment (oxygen and nitrogen atoms in darker grey tones, Mn$_3$ and Mn$_4$ labelled as 3’ and 4’, respectively). Selected residues are labelled.
shifted, but the positions of Mn4 and the Asp170 deviate clearly from the starting structure. We note that, due to the uncertainty in the position of Mn4 and Asp170, the coordination sites of the two coordinated water species and the carboxylate side chain of Asp170 are essentially exchangeable allowing for, e.g. a hydroxide ligand in bridging position between Mn4 and Ca; also bidentate coordination of Asp170 (and a single ligated water species) is conceivable. Chloride ligation is not considered because Cl does not seem to be a first-sphere Mn ligand in the dark-adapted PSI (Haumann et al. 2006).

The resulting structural model suggests that the substrate water molecules bind at or close to Mn4, as proposed first in Ferreira et al. (2004), because (i) only Mn4 is coordinatively not fully saturated by μ-oxo and amino acid ligands and (ii) close to Mn4 there is a cavity which could accommodate two or more additional water molecules thereby facilitating immediate binding of substrate water molecules after dioxygen release.

The assumed oxidation states are: Mn1III, Mn2III, Mn3IV and Mn4IV. This assignment suggests that one of the two water species coordinated to Mn4IV may be a hydroxide. The terminal hydroxide ligand of the Ca ion could be in hydrogen-bonding distance to the phenol oxygen of Tyr160; the terminal H2O ligand is in hydrogen-bonding distance to Asp61; the His337 (not shown) could form a hydrogen bond to the μ2-oxo bridge between Mn2 and Mn3.

\[ \text{Figure 4. Discussed model of the Mn}_4\text{Ca complex and its ligand environment in the S}_1\text{-state. The distances between metal ions are approximately } 2.7 \, \text{Å (Mn2–Mn3, Mn3–Mn4), approximately } 3.2 \, \text{Å (Mn1–Mn2, Mn1–Mn3), approximately } 3.7 \, \text{Å (Mn1–Mn4) and approximately } 3.4 \, \text{Å (several Mn–Ca distances). The bond between the Ca and the terminal carboxylate of Ala344 (and also other Ca-bonds) is uncritical for the model and may be weak or absent. With exception of the Glu354 of the CP43 protein (carboxylate bridging between Mn2 and Mn3; shown but not labelled), all indicated residues are from the D1 protein of PSI. In the } S_0 \rightarrow S_1 \text{ transition, the bridging oxygen labelled by an asterisk is deprotonated; in the } S_2 \rightarrow S_3 \text{ transition the oxygen labelled by a hash is deprotonated and transformed from a weak (Mn–O distance } > 2.4 \, \text{Å) to a strong ligand of Mn1.} \]

\[ \text{Figure 5. Structural model of the Mn}_4\text{Ca complex in the S}_3\text{-state. For clarity, the hypothetical OH}^- \text{ ligand of the calcium, which is depicted in figure 4, and the protonation of the water species ligated to Mn4 are not shown.} \]

Phil. Trans. R. Soc. B (2008)
interrelation between deprotonation of the Mn complex and electron transfer (ET) to the YZ-radical is described by Dau & Haumann (2006, 2007, 2008).

The histidine ligation suggests that Mn1 will be the last Mn ion to reach the MnIV-state (i.e. in the S2→S3 transition). FTIR results suggest that Mn2, which is ligated to Ala344, is oxidized in the S1→S2 transition (Chu et al. 2004) and that Mn4 possibly does not change its oxidation state in the transitions from S0 to S1 (Debus et al. 2005).

We propose that the S0→S1 transition involves MnIII→IV oxidation and deprotonation of the μ-oxo bridge between Mn3 and Mn4 (labelled by an asterisk in figure 4). In the S1→S2 transition, the MnII->IV oxidation is not coupled to deprotonation and structural changes. Up to the S2-state, the hydroxide (labelled by a hash in figure 4) bridging between Mn2 and Ca is only loosely ligated to Mn1 (Mn1–O2 distance > 2.4 Å). In the S2→S3 transition, the five-coordinated MnIII is oxidized and transformed into a six-coordinated MnIV by tighter binding of the O#b, a process associated with deprotonation and proton release. In the thereby formed S3-state complex (figure 5), a MnIII-Ca(μ-O)x cubane is present in a conformation of the MnIII-Ca(μ-O)x core identical to one predicted by quantum chemical calculations (Lundberg & Siegbahn 2004).

As clearly visible in figure 5, the proposed MnI-Ca(μ2-O)n core exhibits striking similarities to motifs found in various modifications of MnII–IV oxyds (layer or tunnel structures with intercalated Ca; Post 1999; Sauer & Yachandra 2002) as well as in synthetic multinuclear Mn (Mukhopadhyay et al. 2003) and Mn–Ca complexes (Mishra et al. 2005). Also, the bidentate carboxylate bridging between Mn ions frequently is found in binuclear Mn sites in proteins (Crowley et al. 2000) as well as in numerous synthetic complexes (Mukhopadhyay et al. 2004).

Here we note that the proposed coordination by the carboxylate side chains of Asp342 (to Mn2) and Glu189 (to Mn1) appears to be in conflict with FTIR results (Strickler et al. 2006, 2007). However, the relation between shifts in vibrational frequencies of coordinating carboxylates and Mn oxidation is only insufficiently understood and could be more intricate than presently assumed. In any event, these and other FTIR results will be crucial touchstones for evaluating than presently assumed. In any event, these and other FTIR results will be crucial touchstones for evaluating

Emphasizing the hypothetical character, here we have suggested for all semi-stable S-states: (i) a structure of the MnIII-Ca(μ-O)x core, (ii) a model of the amino acid environment (largely following Loll et al. (2005)), and (iii) assignments of distinct Mn oxidation states to all the individual Mn ions. Being highly specific, this working model permits discussion, verification, invalidation and modification of distinct aspects by comparison with experimental and theoretical findings.

We thank Dr. Wolfram Meyer-Klaucke and the EMBL staff in Hamburg for their support in XAS data collection at the EMBL beamline (D2) at the DESY and Monika Fünnig for the skilled preparation of XAS samples. Financial support by the Deutsche Forschungsgemeinschaft (DFG) in the framework of the SFB 498 on protein-cofactor interactions and by the Bundesministerium für Bildung und Forschung (BMBF 03SF0318C) in the research consortium ‘Grundlagen für einen biotechnologischen und biomimetischen Ansatz der Wasserstoffproduktion’ is gratefully acknowledged.

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Phil. Trans. R. Soc. B (2008)
Extended-range EXAFS and structural model  H. Dau et al.  1243

Discussion

D. Nocera (MIT, Boston, USA). In your refined S-state cycle, you seem to imply isolated protons isolated from electrons on each step of the Kok cycle owing to the difficulty associated with coupling the classical proton to the quantum mechanical electron. But extensive studies on proton-coupled ET shows that the proton and electron can couple by orthogonalizing the proton and ET coordinates to keep the proton transfer distance short and coupled to a longer ET coordinate. Along your modified Kok cycle are there some H+/e− steps more tightly coupled than others?

H. Dau. We have proposed a basic reaction cycle of photosynthetic oxygen formation which extends the classical Kok cycle (Kok et al. 1970) by explicitly accounting also for proton release (Dau & Haumann 2006, 2007, 2005). In this reaction cycle, four protons and four electrons are removed strictly alternately from the Mn complex, prior to the onset of O–O bond formation. Transfer distance and slowness of the proton movement from the Mn complex to the lumenal bulk excludes that this proton release (to the aqueous phase) and the ET (to the YZ-radical) are directly coupled processes; these processes need to be sequential. However, it is not excluded that the ET to the YZ-radical is directly coupled to proton shifts within the Mn complex (Dau & Haumann 2007, 2005). Specifically, the ET in the S2 → S3 transition or, more precisely, S5 → I6 (Dau & Haumann 2006, 2007, 2005) is a candidate for close coupling between the ET to the YZ-radical, on the one hand, and the shift of a proton within a hydrogen bond at the Mn complex, on the other hand. The sizeable kinetic isotope effect (kD2O/kH2O close to 2) may be compatible with such a proton shift, but appears still to be too small for a rate-limiting influence of a proton tunnelling event. Presently however, there is no clear experimental evidence in favour or against strong coupling of the ET to the YZ-radical to proton shifts within the Mn complex or its ligand environment.

(However, there is reasonably good support for coupling between the ET from and to YZ and the shift of a proton within a strong hydrogen bond between N-His190 and O-Tyr160/161 (Rappaport & Lavergne 1997; Ahlbrink et al. 1998; Hays et al. 1998; Mamedov et al. 1998; Christen & Renger 1999).)

J. Barber (Imperial College London, UK) Bidentate bridging is good but may shift to monodentate at higher valency as emphasized by Gary Brudvig after my talk. If this is the case, then this would open up the requirement for additional non-protein ligands (e.g. water). Have you considered this?

H. Dau. The shift from a monodentate to a bidentate ligation has been proposed to occur in various biological metal centres. We have considered the occurrence of such a carboxylate shift in the water oxidation chemistry of PSII in the past (Dau et al. 1998), but presently we do not think that a carboxylate shift is likely. I have presented a working model of the PSII manganese complex in its S1, S2, S3 and S0-state; carboxylates shifts are not involved. In this model we follow Loll et al. (2005) and assume that each carboxylate which is close to a Mn ion becomes an actual ligand. This results in three bridging carboxylates, a structural motif frequently found in low and high-valent Mn complexes. There remains no monodentate carboxylate which could acquire a bridging position (bidentate bridging between Mn ions) upon oxidation of the Mn4Ca complex in the S-state cycle. In your question you addressed the possibility of a shift from bidentate to monodentate ligation upon oxidation of the Mn4Ca complex. This is not excluded by the proposed model of the S1-state complex per se. However, it remains unclear why Mn oxidation should favour a shift from carboxylate to water ligation.

R. Pace (Australian National University, Canberra, Australia). Where in your structure are the exchangeable substrate water molecules seen by Hillier and his colleagues?

H. Dau. We propose a specific working model for the structure of the Mn4Ca complex of PSII in the four semi-stable S-states which is based on (i) crystallographic results (Ferreira et al. 2004; Loll et al. 2005), (ii) the putative structural modifications by X-ray exposure during crystallographic data collection (Grabolle et al. 2006), (iii) various spectroscopic results on the Mn complex in the S1, S2, S3 and S0-states (Haumann et al. 2005), and (iv) new findings on the S1-state complex recently obtained by our group by means of extended-range EXAFS. In this model the monomeric manganese of Ferreira et al. (2004), which often is denoted as Mn4 is connected by both a μ2-O and a μ3-O bridge to the Mn ions of the remaining Mn3Ca motif. In the working model proposed by us, in the S1-state, all Mn ions besides Mn4 are surrounded by six ligands which are either bridging oxygens or atoms of ligating amino acid residues. The water exchange experiments of Hillier and co-workers (Hillier & Wydrzynski 2004) in conjunction with recent findings of Brudvig and co-workers (Tagore et al. 2006) suggest that the substrate water molecules are not bridging between Mn ions in the S1-state. Thus, our structural model implies that Mn4 represents the binding site of the substrate water molecules. We think that one of these is ligated directly to Mn4, the second one may be ligated to calcium (McEvoy & Brudvig 2006) or even may be an outer-sphere water (i.e. not directly ligated to any metal), as proposed elsewhere (Dau et al. 2001).

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