Focusing the view on nature's water-splitting catalyst

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Nature invented a catalyst about 3 Gyr ago, which splits water with high efficiency into molecular oxygen and hydrogen equivalents (protons and electrons). This reaction is energetically driven by sunlight and the active centre contains relatively cheap and abundant metals: manganese and calcium. This biological system therefore forms the paradigm for all man-made attempts for direct solar fuel production, and several studies are underway to determine the electronic and geometric structures of this catalyst. In this report we briefly summarize the problems and the current status of these efforts and propose a density functional theory-based strategy for obtaining a reliable high-resolution structure of this unique catalyst that includes both the inorganic core and the first ligand sphere.

Keywords: photosystem II; oxygen evolution; manganese cluster; EPR/ENDOR; EXAFS; density functional theory

1. INTRODUCTION

In nature water-splitting is performed within a membrane-bound pigment–protein complex known as photosystem II (PSII). Each PSII complex harvests sunlight by an ‘antenna’ that consists of approximately 200 chlorophyll and several carotenoid molecules. Four specially bound chlorophylls and a pheophytin molecule perform the primary charge separation within the reaction centre that creates a potential of approximately +1.3 V. Each charge separation leads to the oxidation of a metal–oxygen complex, and water is split once four oxidizing equivalents have been accumulated in this water-oxidizing complex (WOC). The different oxidation states of the WOC are generally referred to as S_i states ($S_0, \dots, S_4$), where the index denotes the number of stored oxidizing equivalents relative to the lowest oxidation state of the WOC during the catalytic cycle. For the energetics of this reaction sequence, coupling of electron and proton movements via the protein matrix appears to be of essential relevance

(Hillier & Messinger 2005; Renger & Holzwarth 2005; McEvoy & Brudvig 2006).

Several techniques have contributed to the current picture about the WOC. Historically the greatest impact with regard to the knowledge about the metal ion arrangement in the WOC has been from X-ray absorption fine structure (EXAFS) spectroscopy (Yachandra *et al.* 1996; Yachandra 2005), assisted by vital information from electron paramagnetic resonance (EPR) and ⁵⁵Mn-electron nuclear double resonance (ENDOR) spectroscopy (Britt *et al.* 2000; Kulik *et al.* 2005, 2007), as well as from bioinorganic model chemistry (Mukhopadhyay *et al.* 2004). The equally important information on the ligand sphere has been obtained mostly from site-directed mutagenesis in combination with Fourier transform infrared (FTIR) and EPR spectroscopies (Debus 2005) and by X-ray crystallography (Ferreira *et al.* 2004; Loll *et al.* 2005).

Below we briefly summarize this development and propose a strategy for obtaining a reliable high-resolution structure of the WOC that includes the metal core and the first ligand sphere.

(a) The Mn_4O_xCa cluster of the WOC

The WOC is now known to comprise four manganese ions and one calcium ion that are connected via several μ -oxo bridges (Mn_4O_xCa complex). Solution EXAFS

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revealed already in the 1980s and 1990s the presence of 2–3 Mn–Mn distances at 2.7–2.8 Å and 1–2 Mn–Mn distances at 3.3–3.4 Å. On that basis a set of 11 possible structural motives (some are presented in figure 1*a–e*) were proposed by the Berkeley group (DeRose *et al.* 1994), from which the dimer-of-dimers model was discussed most prominently (figure 1*a*; Yachandra *et al.* 1996).

Mn K-edge EXAFS measurements of PSII samples are normally limited to a range just below the Fe K-edge owing to the natural iron content of PSII samples and because the energy resolution of the Ge X-ray fluorescence detector is not high enough to fully discriminate between Mn and Fe X-ray fluorescence. This restricts the distance resolution to approximately 0.15 Å. By employing a better resolving crystal monochromator that allows us to scan well beyond the Fe K-edge, we recently improved the distance resolution to approximately 0.1 Å. These experiments demonstrate the presence of three short Mn–Mn distances in the range between 2.70 and 2.85 Å (Yano *et al.* 2005*b*).

It was noted early on that the third FT-EXAFS peak contains another contribution that was best assigned to a Mn–Ca interaction at approximately 3.4 Å (Yachandra *et al.* 1993). Since this proposal was controversial, it was proven in many different ways: (i) Mn-EXAFS in samples where Ca was replaced by the better back-scatterer Sr (Sr-PSII) showed an increased third FT peak, while the third peak decreased upon Ca depletion (Latimer *et al.* 1995; Latimer *et al.* 1998), (ii) Sr-EXAFS of (oriented) Sr-PSII membranes revealed 2–3 Sr–Mn interactions that disappeared after NH₂OH treatment (Cinco *et al.* 1998, 2004), (iii) Ca-EXAFS on samples from which all unspecific Ca was removed also allowed the detection of the Ca–Mn interactions (Cinco *et al.* 2002), and (iv) range-extended EXAFS of oriented membranes allowed separation of the Mn–Mn vector at 3.2 Å from the Mn–Ca vector at 3.4 Å (Pushkar *et al.* 2007).

Two anions, namely Cl[−] and bicarbonate (HCO₃[−]), have been proposed to be cofactors in the water-splitting reaction of PSII. While there is compelling biochemical evidence for a functional role of Cl[−] (van Gorkom & Yocum 2005), it is controversial whether it is (in some S states) a direct ligand of the Mn₄O_xCa cluster. Binding of the Cl[−] analogue azide within 5 Å of the Mn₄O_xCa cluster was demonstrated by electron spin-echo envelope modulation (ESEEM) spectroscopy (Yu *et al.* 2005). Similarly, extended EXAFS measurements show an extra peak that may be assigned to Cl[−] (Pushkar *et al.* 2007). On the basis of EXAFS measurements on Br[−] substituted samples, it was recently suggested that Cl[−] is not a direct ligand of the Mn₄ cluster in the S₁ state (Haumann *et al.* 2006). In contrast to Cl[−], no sound evidence exists for HCO₃[−] binding within the WOC, and it is also highly controversial what kind of role it may play during photosynthetic water oxidation (Klimov & Baranov 2001; Stemler 2002; Clausen *et al.* 2005; Hillier *et al.* 2006).

EXAFS on solution samples does not allow extracting the orientations of the Mn–Mn and Mn–Ca vectors. Such information is, however, vital for a further selection between possible models and for

placing such models in the correct orientation within the crystal structure of PSII (see below). Therefore, EXAFS measurements were performed initially on one-dimensionally ordered PSII samples that were obtained by painting and partially drying of PSII membrane fragments (BBY) on plane substrates. This approach allowed, for example, to determine that the Mn–Ca(Sr) vector is close to the membrane normal, while that of the 3.3 Å Mn–Mn vector is close to the plane of the thylakoid membrane (Robblee *et al.* 2001). Once PSII single crystals of large enough dimensions (approx. 0.3 × 0.3 × 0.9 mm) became available, polarized EXAFS was performed on these three-dimensionally ordered samples. This allowed reducing the number of possible geometries for the Mn₄ core to one, while still four different relative positions for Ca remained (Yano *et al.* 2006). Two of these currently most reliable models of the inorganic core of the WOC are presented in figure 1*g,h*.

A different model for the Mn₄O_xCa cluster (figure 1*f*) was previously proposed on the basis of X-ray crystallography (Ferreira *et al.* 2004). However, this model is less reliable owing to (i) limited resolution (3.5 Å) and (ii) severe radiation damage that was shown to occur during X-ray diffraction (XRD) data collection (Yano *et al.* 2005*a*). This radiation damage not only reduces the Mn(III) and Mn(IV) ions of the intact WOC to Mn(II), but also severely alters the structure of the Mn₄O_xCa cluster (Yano *et al.* 2005*a*). However, the pioneering X-ray data of PSII (Zouni *et al.* 2001) indicated the relatively compact arrangement of the Mn ions, and the two latest studies show that the Ca is positioned between the Mn ions and the reaction centre of PSII (Ferreira *et al.* 2004; Loll *et al.* 2005).

All structures shown in figure 1 refer to the S₁ and S₂ states, which may have almost identical geometries. Interestingly, the structure of the cluster changes dramatically upon S₃-state formation (Liang *et al.* 2000; Haumann *et al.* 2005) and is also different in the S₀ state (Robblee *et al.* 2002; Haumann *et al.* 2005). This structural flexibility may be key to the catalytic activity of the WOC.

EPR measurements of the S₂ state were among the first to demonstrate the involvement of Mn in storing the oxidizing equivalents in the WOC (Dismukes & Siderer 1981). In principle, the cw EPR multiline signals of the S₂ and S₀ states do contain all the information about the electronic and geometric structures of these states. In practice, however, it has proven impossible to extract any unique information owing to the large number of undetermined fitting parameters. Significant progress was made on the basis of ⁵⁵Mn-ENDOR measurements on the S₂ state, which allow extracting reliable hyperfine interaction parameters. The analysis of these data strongly disfavoured the dimer-of-dimers model (figure 1*a*), and instead preferred two other models of the initial EXAFS-derived set, which were referred to as 'dangler' models (figure 1*b,c*; Peloquin *et al.* 2000). In the light of extra information from EXAFS and X-ray crystallography, subsequently the more connected dangler model *d* (figure 1) was selected from the initial EXAFS-derived set of structures (Britt *et al.* 2004). This model bears similarities to a structure suggested earlier by Kusunoki and co-workers (Hasegawa *et al.* 1999). In a

Figure 1. (a–h) Proposed models for the inorganic core of the WOC. Red spheres symbolize manganese ions, blue spheres oxygen bridges and green spheres calcium ions. Dashed lines indicate that the bridging motif is unknown. Models redrawn from DeRose *et al.* (1994), Ferreira *et al.* (2004) and Yano *et al.* (2006). Models *g* and *h* correspond to models II and III in Yano *et al.* (2006), respectively.

Figure 2. Proposed spin-coupling schemes for the (a, b, c) S_0 and (d, e, f) S_2 states of the WOC, which are consistent with Q-band ^{55}Mn -ENDOR data (Kulik *et al.* 2005). Red circles indicate the Mn ions, which are labelled A, B, C and D in the same way as in figure 1. The roman numbers in the circles give the formal oxidation states of the Mn ions. Calcium is not included in the spin-coupling schemes since coupling between Mn ions via Ca is assumed to be negligible. The blue numbers give the $\mathcal{J}$ -couplings in cm^{-1} according to $H = -\sum \mathcal{J}_{ik}(\mathbf{S}_i \cdot \mathbf{S}_k)$. The type of line represents the coupling strength: double line, strong antiferromagnetic coupling; single solid line, medium strength antiferromagnetic coupling; and dashed lines, weak ferro- or antiferromagnetic coupling.

separate effort, the ‘funnel’ model (figure 1*e*) was favoured based on EPR data (Carell *et al.* 2002).

Recently Q-band ^{55}Mn -ENDOR measurements were obtained from the S_0 and S_2 states of PSII (Kulik *et al.* 2005, 2007). A thorough analysis shows that the latest EXAFS-derived structural models (figure 1*g,h*) are fully consistent with the ^{55}Mn -ENDOR data (Kulik *et al.*

2007). Our preferred coupling schemes for the S_0 and S_2 states are shown in figure 2(*a, d*). At this level of analysis the coupling schemes for EXAFS models *g* and *h* (figure 1) are indistinguishable, because the two models have the same overall structure and bridging motive.

However, ^{55}Mn -ENDOR is not a sharp knife, and valid solutions can, for example, also be found for the S_0

Figure 3. Ligand environment of model *g* of figure 1 as determined by placing this model into the 3.0 Å crystal structure (Loll *et al.* 2005; Yano *et al.* 2006). (a) Electron density with model *g*. (b) Schematic of the WOC. Blue dashed lines indicate that the displayed amino acids are too far away (more than 3 Å) to be normally considered as ligands. Dashed black lines indicate metal–ligand distances of less than 3 Å. Mn ions are indicated by red spheres, bridging oxygen by grey spheres and Ca by a green sphere. Ligand oxygens are marked orange and ligand nitrogens blue. For further details, see Yano *et al.* (2006).

and S_2 states for the modified dangler (model *d*, figure 1) and the crystallography-derived model *f* (figure 1). These solutions are also displayed in figure 2. Although the agreement known structural changes between S_0 and S_2 and between the proposed structures and the calculated J -couplings is clearly less satisfying for models *g* and *h* (figure 1), the former two cannot be strictly excluded.

(b) The ligands of the Mn_4O_xCa cluster in the WOC

EXAFS spectroscopy does not give any specific information on the ligands of the Mn_4O_xCa cluster. Detailed mutagenesis studies in combination with fluorescence, EPR and FTIR measurements revealed several possible ligands (Debus 2005), many of which were found near the Mn_4O_xCa cluster in the recent XRD studies (Ferreira *et al.* 2004; Loll *et al.* 2005). However, some conflicts exist between these independent approaches (Debus 2005) and also between the two latest crystal structures. Similarly, placing the polarized EXAFS-derived models for the Mn_4O_xCa cluster into the XRD structures (Yano *et al.* 2006) results in unsatisfying metal–ligand distances, coordination numbers and geometries for all four possible models. This is shown for model *g* (figure 1) in figure 3. These discrepancies can be explained by the (i) limited resolution of the X-ray structures and (ii) radiation damage occurring during the XRD measurements, which not only affects the cluster geometry but probably also modifies the ligand positions. These factors also prevent the important localization of the substrate water binding sites.

(c) A DFT-based strategy for deriving a ‘complete’ model of the WOC

As summarized above, reliable information is now available about the positions of the four Mn ions in the WOC: on the basis of polarized single crystal EXAFS, one μ -oxo bridging motive can be inferred, and only four specific options remain for the position of Ca and the

orientation of the Mn_4O_xCa cluster within PSII (Yano *et al.* 2006). In contrast, several uncertainties remain for the important ligand environment and the bridges between Mn and Ca, resulting in a still blurred view of the WOC. Since currently no significant progress is in sight for obtaining radiation damage-free XRD structures of PSII, we decided to embark on a density functional theory (DFT)-based strategy for building a reliable model of the WOC that includes the Mn_4O_xCa cluster and its direct ligands. Previous DFT studies on the WOC have mostly concentrated on model *f* (McEvoy *et al.* 2005; Siegbahn & Lundberg 2006; Sproviero *et al.* 2006, 2007) or on deriving essential mechanistic and structural features of O–O bond formation (Blomberg *et al.* 1997; Siegbahn & Crabtree 1999; Siegbahn 2000, 2006; Siegbahn & Blomberg 2000; Lundberg *et al.* 2003, 2004; Lundberg & Siegbahn 2005).

In our approach towards a better ‘focused’ view on this important catalyst, we start from models of the Mn_4O_xCa cluster as determined by polarized EXAFS spectroscopy on PSII single crystals (Yano *et al.* 2006; models *g* and *h* in figure 1). Because we regard this information to be the most reliable available at present, we initially fix the metal positions. To these models, we add various ligand environments that are consistent with information available from XRD, mutagenesis and FTIR spectroscopy. These ligands are prepositioned by hand and then computationally optimized as described below. After this first energy minimization, we also release for the final geometry optimization the metal positions. The correct structure should reveal itself by minimal structural changes during the final optimization step and by good agreements between experimental spectroscopic parameters from EXAFS, X-ray absorption near edge structure (XANES), EPR and ENDOR and those calculated by DFT methods from the derived models.

We describe below our initial efforts in this direction using a series of selected models.

Figure 4. Comparison of the (a,b) initial structures of models II and IIa, with those obtained (c,d) after constrained optimization. All indicated amino acids are from the reaction centre D1 protein of PSII, with the exception of Glu354, which is a side chain of the inner antenna CP43 protein of PSII. Alanine 344 is the C-terminus of the D1 protein and ligates via its terminal carboxy group. Manganese ions are indicated in purple, oxygen in red, hydrogen in white, calcium in green and carbon in black.

(i) Model construction

All models are built based on the structure of the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster in the S_1/S_2 state as available from EXAFS studies (Yano *et al.* 2006). The positions of the four manganese atoms (A, B, C and D; see figure 1), Ca and five oxygen atoms are available from the supplementary material of the above cited paper.

So far, only EXAFS structures II (g in figure 1), IIa and III (h in figure 1) of Yano *et al.* (2006) were considered. Model I (Yano *et al.* 2006) appears less likely, since the Mn–Ca vectors are not along the membrane normal. In this preliminary work, we have focused on four models that are considered to be promising candidates for the actual structures of the S_1 and S_2 states of PSII. In order to reduce our models to a size that makes extended DFT studies feasible (approx. 80 atoms, including the five metals), acetic acid anions have been considered to represent glutamate, aspartate and the alanine C-terminus of the D1 protein, and only the imidazole ring of the histidine amino acid was included in the models.

The starting structures of all models considered in this study have the following common features: D1-Asp170 is a monodentate ligand of MnA (for labelling of the Mn ions see figure 1); D1-Glu333 is bridging MnA and MnB; CP43-Glu354 is bridging MnB and MnC; D1-His332 ligates MnC; and D1-Glu189 is a bidentate ligand of calcium. Other protein ligands are

placed model specific as explained below. Then, the coordination sphere of each manganese ion was completed (to six coordinate) with water molecules or hydroxyl ions, with the exception of model III-Cl, where one Cl^- is assumed to take the place of one water molecule at MnA (figures 4a,b and 5a,b).

In the starting geometry of model II (figure 4a), D1-Asp342 is a monodentate ligand of MnC, and the C-terminus of D1-Ala344 is bridging MnD and Ca. The main differences between models II and IIa (figure 4a,b) are the position of the calcium ion (as suggested by EXAFS), the replacement of the D1-Asp342 ligand at MnC by a water molecule and that the C-terminus of D1-Ala344 is a bidentate ligand of MnD.

Models II (figure 4a) and III (figure 5a) differ mainly in the position of the μ_3 -oxo bridge that is pointing towards Ca in model II, while in model III it is on the other side of the Mn trimer plane. This affects the way Ca may be bridged to Mn and also the positioning of other ligands such as CP43-Glu354. In the initial geometry of model III, the C-terminus of D1-Ala344 ligates in a bidentate fashion MnD and D1-Asp342 is a monodentate ligand of MnC. For exploring the effects of possible chloride coordination to the $\text{Mn}_4\text{O}_x\text{Ca}$ cluster, we replaced one of the water molecules ligating MnA in model III by Cl^- (model III-Cl; figure 5b). One more difference between models III and III-Cl is that in the latter D1-Asp342 is bridging MnC and MnD.

Figure 5. Comparison of the (a,b) initial structures of models III and III-Cl with those obtained (c,d) after constrained optimization. All indicated amino acids are from the reaction centre D1 protein of PSII, with the exception of Glu354, which is a side chain of the inner antenna CP43 protein of PSII. Alanine 344 is the C-terminus of the D1 protein and ligates via its terminal carboxy group. Manganese ions are indicated in purple, oxygen in red, hydrogen in white, calcium in green, chloride in yellow, carbon in black and nitrogen in blue.

Based on proton release and electrochromic shift measurements, the total charge of the WOC in the S_2 state is likely to be +1 (assuming that it is zero in the S_1 state; Schlodder & Witt 1999). Therefore, protons have been added to the above residues in a way to obtain a total charge of the complex equal to +1. For this charge counting, three manganese atoms are assumed to be in oxidation state IV (d^3) and the fourth one to be in oxidation state III (d^4).

(ii) Calculations details

Geometry relaxations have been performed using the Gaussian v. 03 (Frisch *et al.* 2004) optimizer as implemented in the ORCA package (Neese 2006b), with the BP86 (Perdew 1986; Becke 1988) functional and TZVP (Schäfer *et al.* 1994) basis set for all atoms. In the present work, we have sidestepped the difficult problem of the correct spin coupling of the Mn ions by performing the calculations for a ferromagnetic spin alignment. While this will not be appropriate for property predictions, the effect on the optimized geometries should be very limited (Sigfridsson *et al.* 2001). For the analysis of the electronic structures of

the relaxed geometries, single-point calculations have been performed using the B3LYP (Becke 1993a,b) functional and the TZVP basis set for all atoms. Specifically we obtained the Löwdin charges (Szabo & Ostlund 1989) and spin-density distributions by this method. Total energies were also calculated, but cannot be compared for the different models since they contain different sets of ligands. The obtained distribution of the oxidation states on the manganese centres was not imposed, but is a result of the calculations.

(iii) Results of the constrained optimization

As explained above, the initial constrained optimization was performed for the S_2 state of the WOC with the metal positions fixed to the values determined by EXAFS spectroscopy (Yano *et al.* 2006). The optimization of the positions of the ligand atoms of models II and IIa led to some changes in the coordination environment of the Mn ions as shown in figure 4c,d. For model II the following changes are observed: (i) migration of a proton from one of the water molecules ligated to MnA towards D1-Glu189. This change leaves a hydroxo ligand at MnA in the partially optimized structure. In addition, D1-Glu189

Table 1. Löwdin (Szabo & Ostlund 1989) population analysis of the manganese cluster after partial optimization as obtained from B3LYP/TZVP single-point calculations. (In general, partial charges of all metallic ions are much smaller than their relative formal charges due to the ligand-to-metal electron donation. The partial charge of calcium is only slightly reduced revealing the more ionic nature of the Ca–ligand bonds. This can also be seen from the Ca–ligand distances that are on average 0.2–0.5 Å longer than Mn–ligand distances (pdb coordinates of the optimized structures are given in the electronic supplementary material). The spin-density distributions show that about four unpaired electrons ($d^4 \rightarrow \text{MnIII}$) are located on the Mn–D for models II, IIa and III, and only three unpaired electrons can be found (on average) on each of the three other magnetic centres. However, for model III–Cl, it is Mn–A which has the four unpaired electrons, instead.)

	charge distribution					spin-density distribution			
	A	B	C	D	Ca	A	B	C	D
II	0.66	0.64	0.66	0.78	1.52	2.95	2.97	2.96	3.85
IIa	0.64	0.64	0.63	0.82	1.53	2.87	2.94	2.91	3.85
III	0.65	0.63	0.66	0.78	1.49	2.87	2.98	2.96	3.84
III–Cl	0.61	0.62	0.62	0.71	1.49	3.86	2.94	2.89	3.00

changes from being a bidentate ligand of Ca to being a monodentate one. (ii) The position of CP43–Glu354 changes in a way that it no longer bridges MnB, MnC and MnD, but is almost a monodentate ligand of MnB, with a much larger distance (2.4 Å) to MnD. This leaves MnC, and practically also MnD in a five-coordinate geometry.

The latter change occurs in a similar fashion also during the constrained optimization of model IIa, only that the ligation of CP43–Glu354 to MnD remains intact (figure 4d). However, for model IIa the C-terminus of D1–Ala344 changes from being a bidentate ligand to a monodentate ligand of MnD, which is consistent with conclusions from FTIR spectroscopy (Chu *et al.* 2004). Therefore, MnD is again in a five-coordinate ligand environment after constrained geometry optimization.

As in the case of model III, the C-terminus of D1–Ala344 changes during the constrained optimization from being a bidentate to a monodentate ligand of a five-coordinate MnD (figure 5c). In contrast, ligation of CP43–Glu354 is stable for models III and III–Cl and MnC remains six coordinate. Model III shows the formation of an H-bond between the water molecule bridging MnD and Ca and one of the two μ -oxo bridges between MnA and MnB, which brings these two oxygen atoms into a suitable geometry (O–O distance of 2.48 Å) for later O–O bond formation. Interestingly, in case of Cl^- binding to MnA (Model III–Cl in figure 5b,d) the proton is transferred from this hydrogen-bonded water molecule to the μ -oxo bridge, which elongates the MnA–O and MnB–O distances.

The constrained optimizations of both II and IIa (S_2 state) converged towards wave functions characterized by the oxidation states $\text{Mn}_{\text{ABCD}}(\text{IV}, \text{IV}, \text{IV}, \text{III})$ on manganese A, B, C, D, respectively (table 1). The partial geometry relaxations of models III and III–Cl lead to different charge and spin-density distributions relative to each other. Model III also presents the oxidation states $\text{Mn}_{\text{ABCD}}(\text{IV}, \text{IV}, \text{IV}, \text{III})$, while for III–Cl the oxidation-state distribution is $\text{Mn}_{\text{ABCD}}(\text{III}, \text{IV}, \text{IV}, \text{IV})$. The preference for finding Mn(III) in position D is very likely the consequence of MnD being five coordinate in models II, IIa and III after the constrained geometry optimization. This may explain the difference between this result and the manganese oxidation-state assignment for the S_2 state presented in

coupling scheme *d* (figure 2). It is probable that with a modified ligand environment a different assignment is obtained. In the case of model III–Cl, for example, MnA attains oxidation state III and MnD is found in a six-coordinate environment.

(iv) Full optimization

The complete relaxation of the coordinates of the four models considered led to further structural changes, but the oxidation-state distributions and the coordination scheme of all ligands remained the same as after the partial optimization, i.e. no further ligand migrations were observed (figure 6).

Table 2 shows Mn–Mn and Mn–Ca distances before and after full optimization. In general the Ca–Mn distances changed more significantly during full optimization than the Mn–Mn distances. It is therefore remarkable that model IIa almost perfectly reproduces the experimentally determined values of the Mn–Ca distances. Model IIa gives also a fair reproduction of the Mn–Mn distances, with the exception of the MnB–MnC distance being 0.1 Å too long and the MnB–MnD distance being 0.14 Å too short after full optimization. A difference of approximately 0.1 Å in the metal–metal distances is, however, probably within the precision limits of the present DFT methodology. Better agreement with experimental values than to approximately 0.1 Å is not to be expected owing to the neglect of the protein environment, the chosen ferromagnetic spin alignment and the intrinsic error of the DFT methods. Another point that needs to be considered in the future is the possibility for protonations of μ -oxo bridges. Based on our experience of the past decade (Neese 2006a), metal–oxo bonds will be predicted with significantly higher precision (approx. 0.02–0.03 Å) while the bonds to the more weakly bound terminal water ligands will typically be overestimated by 0.05–0.1 Å by the BP86/TZVP combination.

The MnA–MnB and MnC–MnD distances are well reproduced by all models (with the exception of the MnA–MnB distance in model III–Cl). In contrast, model II is the only model that provides a good fit for the MnB–MnD (3.3 Å) distance. For all fully optimized models, the MnB–MnC distance is 0.07–0.14 Å too long when compared with the experimental data, and the closest match is found with models III and IIa.

Figure 6. Molecular structures of (a) II, (b) IIa, (c) III and (d) III-Cl models after constrained optimization (red) and after full optimization (blue). Manganese and calcium ions are represented by spheres. All hydrogen atoms are removed for clarity.

Table 2. Mn–Mn and Ca–Mn distances (Å) before (EXAFS) and after full geometry optimization (DFT) of the four considered models. (The initial values are those determined by single crystal EXAFS spectroscopy (Yano *et al.* 2006). Distances that changed by 0.1 Å or less during full optimization are shown in *italics*. MnA–MnC and MnA–MnD distances are in all cases between 5 and 6 Å.)

		A–B	B–C	C–D	B–D	Ca–A	Ca–B	Ca–C	Ca–D
II	EXAFS	2.72	2.70	2.80	3.29	4.41	3.75	3.41	3.41
	DFT	2.75	2.84	2.83	3.29	3.68	3.71	4.08	3.78
IIa	EXAFS	2.72	2.70	2.80	3.29	3.61	3.40	4.36	3.39
	DFT	2.73	2.80	2.80	3.15	3.52	3.43	4.27	3.42
III	EXAFS	2.72	2.72	2.80	3.25	4.38	3.73	3.40	3.40
	DFT	2.70	2.79	2.72	3.50	3.98	3.87	3.71	3.53
III-Cl	EXAFS	2.72	2.72	2.80	3.25	4.38	3.73	3.40	3.40
	DFT	2.89	2.84	2.77	3.63	4.29	3.70	3.63	3.39

2. DISCUSSION AND CONCLUSIONS

The geometry optimized models presented represent a preliminary account of our efforts towards building a reliable model for the WOC. It cannot be expected that such an approach will easily lead to a unique answer concerning the detailed geometric structures of the various S_i states in the reaction cycle of PSII. We therefore do not compare these preliminary data in detail with data from the literature or our own proposals.

However, present calculations do (i) provide some hints for directions that are worthwhile for further exploration and (ii) indicate that models II, IIa and III, which were proposed on the basis of polarized EXAFS on PSII single crystals, provide stable and chemically reasonable structures for the inorganic core of the WOC. They also reveal that relatively small differences in the ligand environments of models II, IIa and III, III-Cl have significant effects on the metal–metal distances and the spin-density distributions. This provides a basis for further optimizations, for which possibly also a slightly

extended protein environment needs to be included, for example, via the quantum mechanics/molecular mechanics (QM/MM) method. This can be nicely seen by the effect that Cl^- binding to MnA has on the Mn–Ca distances, which are relatively remote from the Cl^- binding site (compare model III and III-Cl in table 2). For the time being, models IIa and III appear to provide the best overall fit to the geometry, and small additional changes to the ligand sphere may lead to an even better agreement of all models with the available structural data for the S_1 (S_2) state.

Once a good agreement of all metal–metal distances is achieved, calculations of polarized EXAFS and XANES spectra and of EPR/ENDOR parameters will be performed to further test the reliability of such pre-selected structures. For electronic structure calculations, the complex problem of the correct spin coupling in the cluster needs to be solved—a task that is far from trivial and requires the estimation of reasonable pairwise exchange couplings between the manganese ions in

conjunction with the treatment of double exchange phenomena owing to the fact that the cluster is in a mixed valence state. These are the subjects of ongoing efforts in our laboratories.

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Discussion

R. Pace (Australian National University, Canberra, Australia). Would the nitrogen ¹⁴N coupling from His 332 to MnC in your model be consistent with that seen by Dave Britt?

J. Messinger. We have to calculate that in detail, but at first sight this appears to be fully consistent.

A. Boussac (CEA, Saclay, France). How do you explain the high-spin states in the S₂ state?

J. Messinger. I have only a preliminary answer to your question. Our Q-band ⁵⁵Mn-ENDOR experiments are performed, as most other EPR/ENDOR studies, with a few percent of methanol, which suppresses in spinach BBY samples the formation of the higher-spin states ($g \geq 4$ EPR signals). Experiments in absence of alcohols give somewhat different, more complex results (Peloquin et al. 2000; electronic supporting information to Kulik et al. 2007) that may reflect a mixture of states and therefore require detailed future analysis.

We briefly tried your suggestion of a valence swap between two Mn ions (Charlot et al. 2005). This appears to work reasonably well, but further detailed studies will be required for a more definitive answer.

R. D. Britt (UC Davis, California, USA). Do you know that you cannot see the S₀ EPR signal in spin echo detection?

J. Messinger. In presence of methanol we do see the S₀ EPR multiline signal with spin echo detection (Kulik et al. 2005). We have not yet tried the same experiment in absence of methanol, but I expect to see a S₀ EPR signal also under these conditions.

The reason for this expectation is considered to be the following: for cw X-band, the S₀ signal is usually not seen in absence of methanol, probably because of a significant broadening of the hyperfine lines under these conditions. However, with high sample concentrations we did observe a broad, featureless signal around $g=2$ for the S₀ state in samples containing no methanol (Messinger et al. 1997). Similarly, *Thermosynechococcus elongatus* preps display an S₀ EPR signal in absence of methanol (Boussac et al. 1999).

C. Dismukes (Princeton University, USA). Do you know how the ⁵⁵Mn quadrupole coupling will influence your conclusions about the exchange couplings (J_{ij}) between Mn ions and the resulting prediction of electronic spin states? This question stems from your choice to ignore these interactions in your simulations. J. Messinger. When we simulate our experimental ⁵⁵Mn-ENDOR spectra we derive four effective hyperfine interaction tensors, $A_{i,iso}$. These form the basis for the further analysis, in which we derive for different spin-coupling models possible exchange couplings between the individual Mn ions. During our initial simulation attempts we also tried including nuclear quadrupole interactions (NQI). This improved somewhat the quality of the simulations, but did not significantly change the $A_{i,iso}$ values (less than 10%). This is expected, since NQI can broaden the ENDOR transitions of the four Mn ions, but it does not shift the centre of the individual spectra. This is confirmed by the good agreement between our Q-band derived $A_{i,iso}$ values for the S₂ state (NQI excluded during simulations) and those derived previously by the Britt group on the basis of X-band ⁵⁵Mn-ENDOR spectra by including NQI during the simulations.

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