

High-resolution structure of the photosynthetic Mn₄Ca catalyst from X-ray spectroscopy

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The application of high-resolution X-ray spectroscopy methods to study the photosynthetic water oxidizing complex, which contains a unique hetero-nuclear catalytic Mn₄Ca cluster, is described. Issues of X-ray damage, especially at the metal sites in the Mn₄Ca cluster, are discussed. The structure of the Mn₄Ca catalyst at high resolution, which has so far eluded attempts of determination by X-ray diffraction, X-ray absorption fine structure (EXAFS) and other spectroscopic techniques, has been addressed using polarized EXAFS techniques applied to oriented photosystem II (PSII) membrane preparations and PSII single crystals. A review of how the resolution of traditional EXAFS techniques can be improved, using methods such as range-extended EXAFS, is presented, and the changes that occur in the structure of the cluster as it advances through the catalytic cycle are described. X-ray absorption and emission techniques (XANES and K β emission) have been used earlier to determine the oxidation states of the Mn₄Ca cluster, and in this report we review the use of X-ray resonant Raman spectroscopy to understand the electronic structure of the Mn₄Ca cluster as it cycles through the intermediate S-states.

Keywords: X-ray absorption fine structure; manganese; oxygen-evolving complex; polarized X-ray absorption fine structure; photosystem II; photosystem II single crystals

1. INTRODUCTION

Life on earth relies on oxygen, and only owing to the constant regeneration of oxygen through photosynthetic water oxidation by green plants, cyanobacteria and algae is it abundant in the atmosphere. The structure of the catalytic Mn₄Ca complex, which oxidizes H₂O to O₂ and is part of a multi-protein membrane system known as photosystem II (PSII), has been the subject of intense study ever since Mn was identified as an essential element for oxygenic photosynthesis (Wydrzynski & Satoh 2005). Water oxidation in PSII is a stepwise process wherein each of four sequential photons absorbed by the reaction centre powers the advance of the oxygen-evolving complex (OEC) through the S-state intermediates S₀ through S₄ (Kok *et al.* 1970). Upon reaching the S₄ state, the complex releases O₂ and returns to S₀. The critical questions related to this process are the structural and electronic state changes in the Mn₄Ca complex as the OEC proceeds through the S-state cycle. The recent results from our laboratory that address these

questions, using polarized X-ray absorption fine structure (EXAFS) of single crystals of PSII (Yano *et al.* 2005a; Yano *et al.* 2006), range-extended EXAFS (Yano *et al.* 2005b; Pushkar *et al.* 2007) and X-ray Raman spectroscopy or resonant inelastic X-ray spectroscopy (RIXS; Glatzel *et al.* 2004; Glatzel *et al.* 2005), were presented at the Royal Society Discussion Meeting on ‘Revealing how nature uses sunlight to split water’ and are reviewed here.

2. STRUCTURE OF THE Mn₄Ca CLUSTER IN PHOTOSYNTHESIS

(a) *Single crystal X-ray spectroscopy*

A key to understanding the mechanism of water oxidation by the OEC in PSII is the determination of the structure of the Mn₄Ca cluster, which has eluded both Mn X-ray absorption fine structure (EXAFS; Penner-Hahn 1998; Dau *et al.* 2001; Yachandra 2005; Sauer *et al.* 2008) and X-ray diffraction (XRD) studies (Zouni *et al.* 2001; Kamiya & Shen 2003; Ferreira *et al.* 2004; Loll *et al.* 2005) until now. Availability of PSII single crystals and the X-ray crystallographic studies of PSII have opened up the possibility of using spectroscopic studies of single crystals to obtain a detailed

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One contribution of 20 to a Discussion Meeting Issue ‘Revealing how nature uses sunlight to split water’.

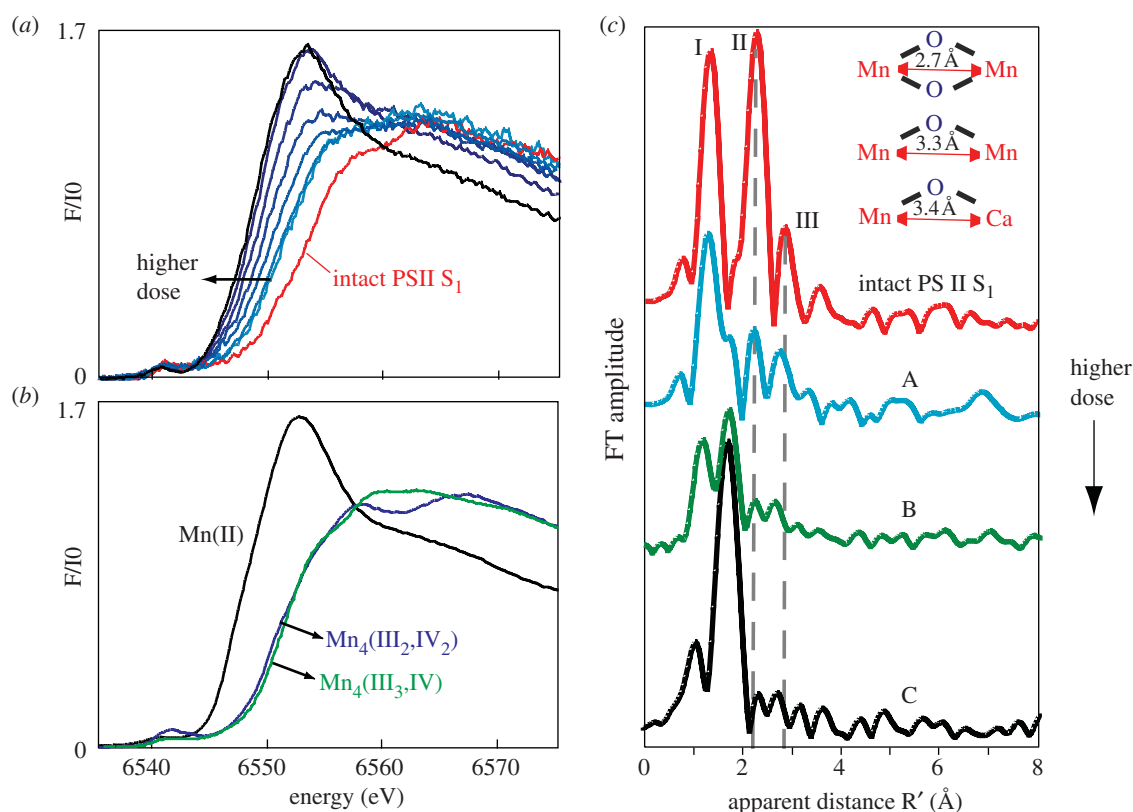


Figure 1. Mn XANES and EXAFS of single crystals of PSII from *Thermosynechococcus elongatus* as a function of X-ray dose. (a) PSII single crystal: as the X-ray dose increases, Mn in PSII normally present as Mn₄(III₂, IV₂) is reduced to Mn(II) as seen by the changes in XANES spectra. (b) Mn models: the Mn XANES from Mn complexes in formal oxidation states II, III and IV. (c) The changes in the EXAFS spectra show that the three Fourier peaks characteristic of Mn-bridging-oxo, Mn-terminal and Mn–Mn/Ca interactions (dashed vertical line) are replaced by one Fourier peak characteristic of a Mn(II) environment. The FT marked C is from a sample given approximately the average dose in the XRD studies. The FT on top from an intact PSII is characteristic of samples used in the X-ray spectroscopy experiment.

high-resolution structure of the catalytic site of water oxidation in PSII.

(i) Damage by X-rays: a case study for metalloprotein crystallography

In order to determine a safe level of X-ray dose for our single crystal X-ray spectroscopy studies, we conducted a comprehensive survey of the effects of radiation dose, energy of the X-rays and temperature on the structure of the Mn₄Ca cluster in single crystals and solutions of PSII. These studies (Yano *et al.* 2005a) have revealed that the conditions used for structure determination by XRD methods are damaging to the metal site structure as monitored by X-ray absorption near edge structure (XANES) and EXAFS spectroscopy, for both cyanobacteria and spinach at room and lower temperatures (Yano *et al.* 2005a; Grabolle *et al.* 2006). With the advent of third generation synchrotron sources, the intensity of X-rays available for crystallography has increased considerably, and X-ray damage to biomolecules has been a continuing concern. This is a well-recognized problem, and various steps have been taken to minimize such damage (Garman 2003). In most crystallography studies, it is the loss of diffractivity as evidenced by loss of resolution that is used as a measure of X-ray damage (Henderson 1990). However, as shown in our study (Yano *et al.* 2005a), this method is not sensitive to localized damage as is prevalent in redox-active metal-containing proteins. We used *in situ*

XANES and EXAFS to show that for the Mn₄Ca cluster in PSII the damage to the metal site precedes the loss of diffractivity by more than an order of magnitude of X-ray dose. This case study demonstrates that such *in situ* evaluation of the structural intactness of the active site(s) by spectroscopic techniques can be critical to validate structures derived from crystallography. Since structure–function correlations are routinely made using the structures determined by X-ray crystallography, it is important to know that the metal site structures are indeed intact. This is particularly important for radiation-sensitive metalloproteins.

Although X-ray crystallography studies of PSII have added valuable information to our knowledge about the location of the Mn₄Ca cluster, at present there are serious discrepancies among the structural models for the Mn₄Ca complex derived from these studies, and inconsistencies with spectroscopic data. This disagreement is probably a consequence of both X-ray-induced damage to the catalytic metal site and differences in the interpretation of the electron density at the presently available resolution (3.0–3.8 Å; Zouni *et al.* 2001; Kamiya & Shen 2003; Ferreira *et al.* 2004; Loll *et al.* 2005).

The Mn XANES data from PSII single crystals show that, following X-ray doses characteristic of the XRD measurements, Mn are reduced to Mn(II) from Mn₄(III₂,IV₂) present in the S₁ state (figure 1, left). Moreover, the EXAFS spectrum changes significantly,

from one that is characteristic of a multinuclear oxo-bridged Mn₄Ca complex to one that is typical of mononuclear hexa-coordinated Mn(II) in solution (figure 1, right; Yano *et al.* 2005a). These X-ray spectroscopy results present clear evidence that there are no specific metal bridging or metal–metal interactions, as shown by the disappearance of the Fourier peaks that are characteristic of Mn-bridging oxo, and Mn–Mn/Mn–Ca interactions with di- μ -oxo or mono- μ -oxo bridges, and by the appearance of Mn–O distances characteristic of Mn(II)–O ligand bond lengths. The density seen in the XRD results is in all probability from the Mn(II)-hexa-coordinated ions that are distributed in the binding pocket, presumably bound to carboxylate ligands and free water, leading to a distribution of metal-binding sites and the resultant unresolved electron density. The metal ions have not necessarily moved away from the site, it is just that they are all bound in a distribution of sites, without the presence of di- or mono- μ -oxo-bridged Mn–Mn or Mn–Ca units.

These studies reveal that the conditions used for structure determination by X-ray crystallography cause serious damage, specifically to the metal site structure, and they provide quantitative details. The results show furthermore that the damage to the active metal site occurs at a much lower X-ray dose than that causing the loss of diffractive power of the crystals as established by X-ray crystallography. The damage is significantly higher at wavelengths used for anomalous diffraction measurements and is much lower at liquid He temperatures (10 K) compared with 100 K, where the crystallography experiments were conducted. For future X-ray crystallography work on the Mn₄Ca complex, it will therefore be imperative to develop protocols that mitigate the X-ray-induced damage. More generally, these data show that in redox-active metalloproteins careful evaluations of the structural intactness of the active site(s) are required before structure–function correlations can be made on the basis of high-resolution X-ray crystal structures.

(ii) Structure of the Mn₄Ca complex from polarized EXAFS of PSII crystals

PSII membranes can be oriented on a substrate such that the membrane planes are roughly parallel to the substrate surface. This imparts a one-dimensional order to these samples; while the z axis for each membrane (collinear with the membrane normal) is roughly parallel to the substrate normal, the x and y axes remain disordered (Rutherford 1985). Exploiting the plane-polarized nature of synchrotron radiation, spectra can be collected at different angles between the substrate normal and the X-ray e -vector. The dichroism of the absorber–backscatterer pair present in the oriented samples is reflected in, and can be extracted from, the resulting X-ray absorption spectra (George *et al.* 1989; Mukerji *et al.* 1994; Dau *et al.* 1995; Dau *et al.* 2001; Cinco *et al.* 2004). The EXAFS of the oriented PSII samples exhibits distinct dichroism, from which we have deduced the relative orientations of several interatomic vector directions relative to the membrane normal and derived a topological representation of the metal sites in the

OEC. However, because the samples are ordered in only one dimension, the dichroism information is available only in the form of an angle with respect to the membrane normal. For EXAFS measurements, this means that the absorber–backscatterer vectors can lie anywhere on a cone defined by the angle the vector forms with the membrane normal.

Further refinement can be performed if samples with three-dimensional order, i.e. single crystals, are examined instead of oriented membranes (Scott *et al.* 1982; Flank *et al.* 1986). This is possible because the EXAFS amplitude is proportional to approximately $\cos^2\theta$, where θ is the angle between the X-ray E vector and the absorber–backscatterer vector. These studies have been able to significantly expand the X-ray absorption spectroscopic information available for these systems over what is gleaned from studies of isotropic samples. We have developed the methodology for collecting single crystal X-ray absorption spectroscopy (XAS) data from PSII. XAS spectra were measured at 10 K using a liquid He cryostat or cryostream, and an imaging plate detector placed behind the sample was used for *in situ* collection of diffraction data and determination of the crystal orientation.

We have reported the polarized EXAFS spectra of oriented PSII single crystals, which result in a set of four similar high-resolution structures for the Mn₄Ca cluster (figure 2a; Yano *et al.* 2006), which were derived from simulations of single crystal polarized spectra based on the experimentally observed dichroism. These structural models are dependent only on the spectroscopic data, and are independent of the electron density from XRD studies. The structure of the Mn₄Ca cluster from the polarized EXAFS study of PSII single crystals is unlike either the 3.0 (Loll *et al.* 2005) or 3.5 Å (Ferreira *et al.* 2004) resolution X-ray structures, and other previously proposed models. The structure of the Mn₄Ca cluster favoured in our study contains structural features that are unique and are likely to be important in facilitating water-oxidation chemistry.

In order to discriminate between symmetry-related structural models that are possible owing to the presence of a non-crystallographic C₂ axis, the cluster was placed within PSII taking into account the overall shape of the electron density of the metal site and the putative ligand information from X-ray crystallography, including infrared and electron paramagnetic resonance (EPR) studies. The centre of mass of the structural models was translated to the centre of electron density attributed to the Mn₄Ca cluster in the 3.0 Å XRD structure (Loll *et al.* 2005) and the models were not optimized with respect to the electron density, or rotated with respect to the crystal axes, as that would change the dichroic characteristics. Of the four options shown in figure 2a, it is possible to rule out Model I on the basis of oriented membrane EXAFS data (Cinco *et al.* 2004; Pushkar *et al.* 2007). Model II, IIa and III in the putative ligand environment are shown in figure 2b. However, we caution that the ligand positions are tentative because they are likely to be affected by radiation damage, as are the positions of the metals atoms from XRD (Yano *et al.* 2005a; Grabolle *et al.* 2006).

The polarized EXAFS study of PSII single crystals demonstrates that the combination of XRD and polarized EXAFS has several advantages for unravelling

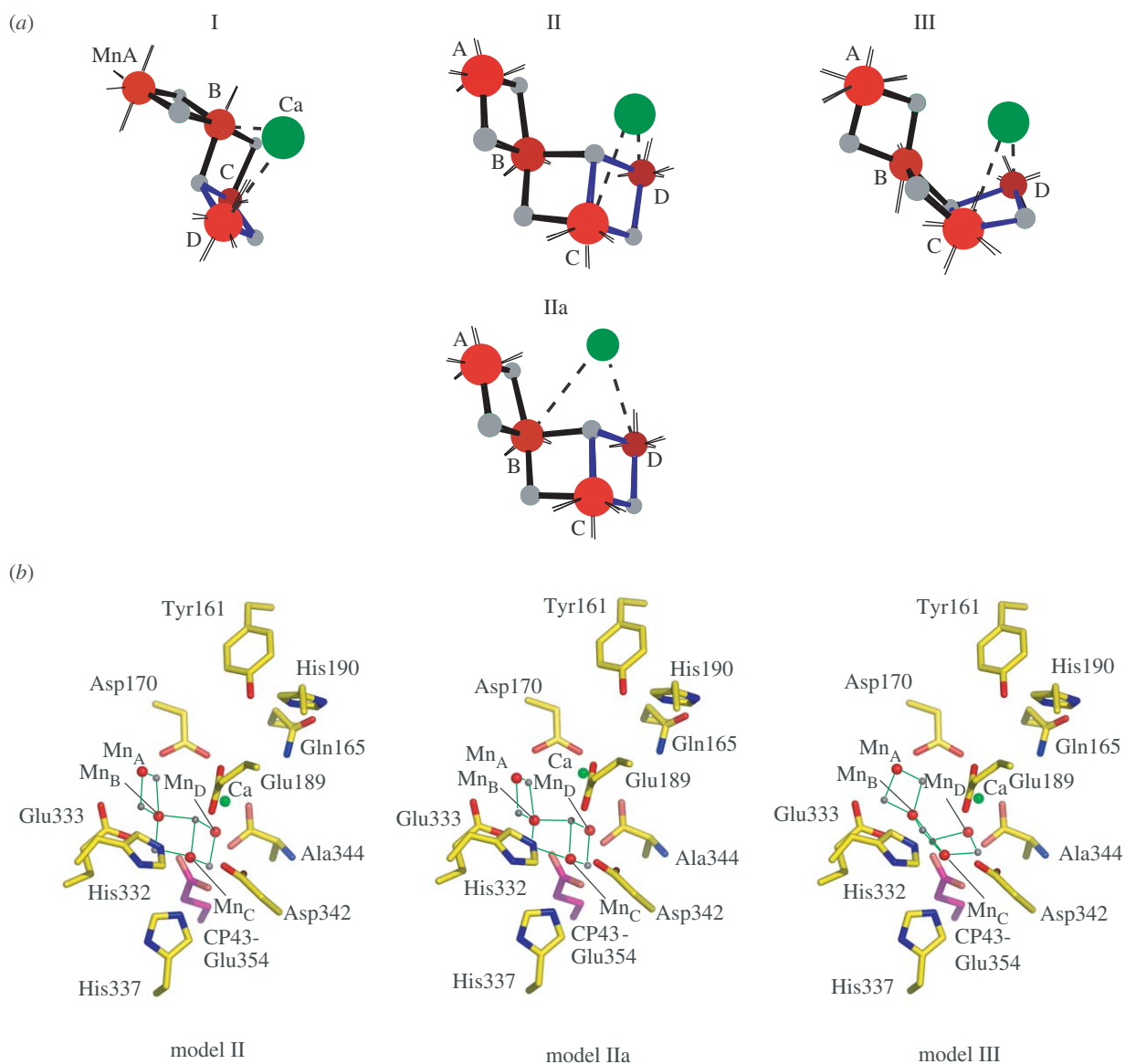


Figure 2. (a) The structural models determined from the polarized EXAFS spectra of oriented PSII single crystals from *T. elongatus*. The distance between Mn_A – Mn_B , Mn_B – Mn_C is approximately 2.7 Å, Mn_C – Mn_D is approximately 2.8 Å, and Mn_B – Mn_D is approximately 3.2 Å. There are at least two Ca–Mn distances of approximately 3.4 Å (Yano *et al.* 2006). (b) The structural models II, IIa and III are placed in the ligand environment from the XRD data of Loll *et al.* at a resolution of 3.0 Å. The centre of mass was translated to the centre of electron density attributed to the Mn_4Ca cluster in the XRD structure without any rotation (Loll *et al.* 2005).

structures of X-ray damage-prone, redox-active metal sites in proteins. XRD structures at medium resolution are sufficient to determine the overall shape and placement of the metal site within the ligand sphere, and refinement using polarized EXAFS can provide accurate metal–metal/ligand vectors. In addition, we are extending the study to determine the structure of the Mn_4Ca cluster in the intermediate S-states of the OEC, which are difficult to study with XRD at high resolution.

(b) Range-extended X-ray absorption spectroscopy

EXAFS spectra of metalloprotein samples are normally collected in X-ray fluorescence yield by electronically windowing the $K\alpha$ emission (2p to 1s) from the metal atom (Scott 1984; Cramer 1988; Yachandra 1995). The solid state detectors used over the past decade have a resolution of 150–200 eV, full width at half maximum

(FWHM), making it impossible to fully discriminate the fluorescence of the absorbing atom (Mn in our case for PSII) from that of the following element (Fe in PSII) in the periodic table. The distances from Mn that can be resolved in a typical EXAFS experiment are given by $\Delta R = \pi/2\Delta k$, where k is the energy of the Mn photoelectron. Unfortunately, owing to the presence of Fe in the PSII samples we have been limited to a Δk of approximately 12 \AA^{-1} , which places an inherent limit on the resolution of Mn distances to approximately 0.14 Å. We have overcome this limitation by using a high-resolution crystal monochromator (Bergmann & Cramer 1998), which has a resolution of 1 eV to collect EXAFS spectra well beyond the Fe K-edge to $k = 15 \text{ \AA}^{-1}$, thus improving the distance resolution to 0.09 Å (figure 3a). We have recently shown the feasibility of the range-extended EXAFS method and collected data without interference from Fe in the sample (Yano *et al.* 2005b).

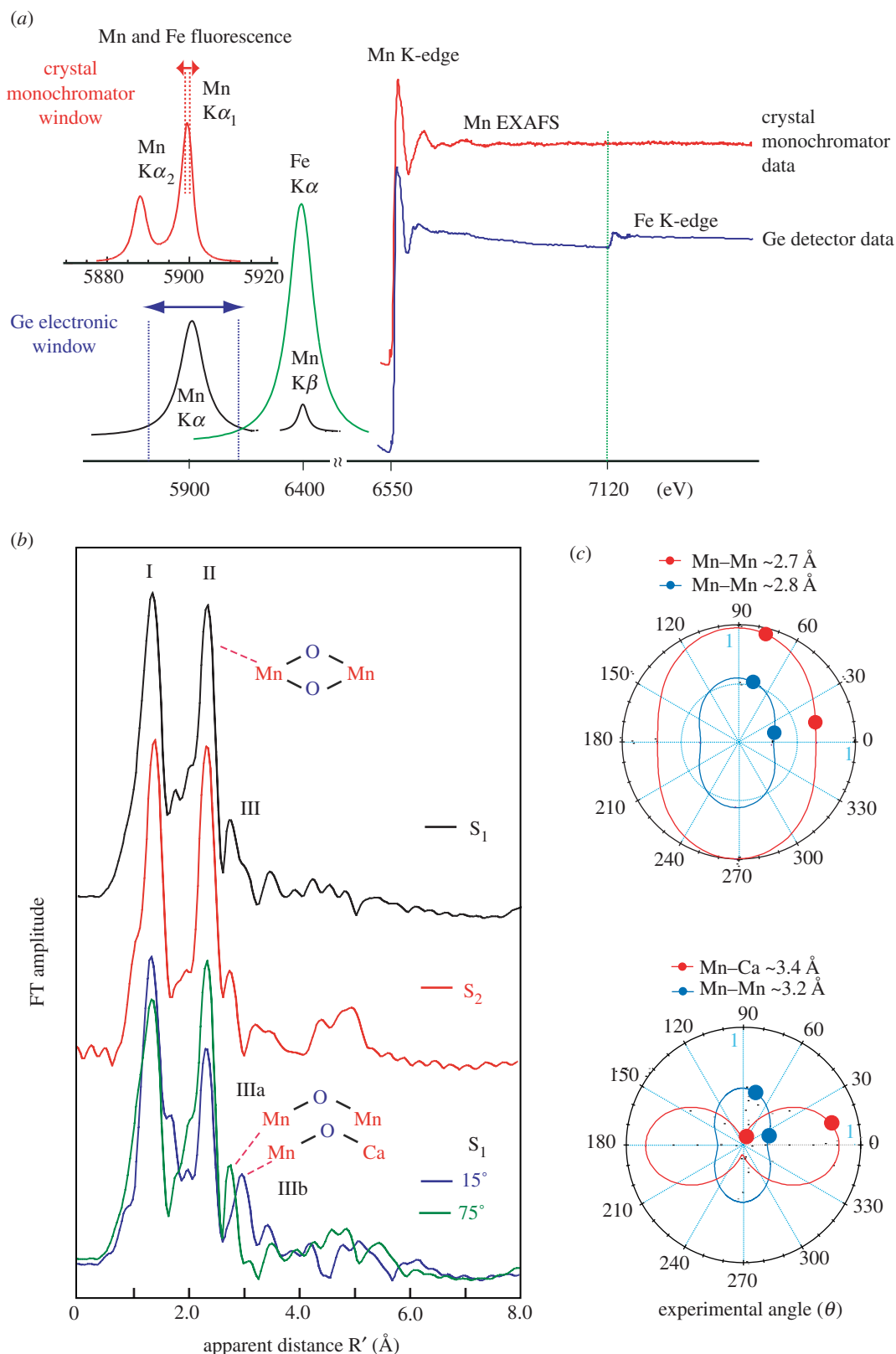


Figure 3. (a) A schematic of the detection scheme. Mn and Fe $K\alpha_1$ and $K\alpha_2$. The multi-crystal monochromator with approximately 1 eV resolution is tuned to the $K\alpha_1$ peak (red). The fluorescence peaks broadened by the Ge-detector with 150–200 eV resolution are shown below (blue). Right: The PSII Mn K-edge EXAFS spectrum from the S_1 state sample obtained with a traditional energy-discriminating Ge-detector (blue) compared with that collected using the high-resolution crystal monochromator (red). Fe present in PSII does not pose a problem with the high-resolution detector (the Fe edge is marked by a green line). (b) Fourier transform (FT) of Mn K-edge EXAFS spectra from isotropic solution and oriented PSII membrane samples in the S_1 , S_2 and oriented S_1 state obtained with a high-resolution spectrometer (range-extended EXAFS). Angles indicate orientation of the membrane normal with respect to the X-ray e -field vector. The k range was 3.5–15.2 \AA^{-1} . (c) Polar plot of the X-ray absorption linear dichroism of PSII samples in the S_1 state. The N_{app} values (solid circles) for oriented membranes are plotted at their respective detection angles (θ). The best fits of N_{app} versus θ to the equation in the text are shown for the various Mn–Mn vectors as solid lines that take into account the experimentally determined mosaic spread of $\Omega = 20^\circ$.

We can now resolve two short Mn–Mn distances in the S₁ and S₂ states, while earlier we could discern only one distance of approximately 2.7 Å. We have also collected range-extended polarized EXAFS data using oriented PSII membranes, and it is quite clear that the heterogeneity is more obvious; the Mn–Mn distance is approximately 2.7 or 2.8 Å depending on the angle the X-ray *e*-vector makes with the membrane normal. Interestingly, for the first time we were able to resolve the Fourier transform (FT) peak at approximately 3.3 Å into one Mn–Mn vector at 3.3 Å and two Mn–Ca vectors at 3.4 Å, which are aligned at different angles to the membrane normal (Pushkar *et al.* 2007).

In figure 3*b* FTs of the range-extended EXAFS for PSII in isotropic solution and in oriented membranes in S₁ state are compared. Peaks are labelled I, II, III corresponding to the shells of backscatterers from the Mn absorber. Peak I contains Mn–O bridging and terminal interactions, and peak II corresponds to di- μ -oxo-bridged Mn–Mn moieties, peak III has information about mono- μ -oxo-bridged Mn–Mn and Mn–Ca interactions. Increased spectral resolution results in the detection of the orientation dependence of peaks II and III.

Figure 3*c* shows polar plots of the N_{app} plotted (solid circles) with respect to detection angle (θ). By fitting the angle dependence of the amplitude (N_{app}) to equation (2.1),

$$N_{\text{app}}(\theta) = N_{\text{iso}} + \frac{1}{2}N_{\text{iso}}(3 \cos^2\theta - 1)(3 \cos^2\phi - 1)I_{\text{ord}}, \quad (2.1)$$

the approximate number of backscatterers (N_{iso}) and the average orientation $\langle\phi\rangle$ for that shell of scatterers can be determined. Analysis of the orientation dependence of the approximately 2.7 Å Mn–Mn vector results in two Mn–Mn interactions at an average angle of $\langle\phi\rangle = 61 \pm 5^\circ$, with respect to the membrane normal. The approximately 2.8 Å Mn–Mn vector has one Mn–Mn interaction at an angle of $\langle\phi\rangle = 64 \pm 10^\circ$ with respect to the membrane normal. In this way, we independently derived that the three Mn–Mn distances of approximately 2.7 Å and approximately 2.8 Å are present in a 2 : 1 ratio in PSII samples in the S₁ state.

The range-extended EXAFS method allowed us to resolve the complex nature of the peak III containing at least two peaks, IIIA and IIIB, having different distances of 3.2 and 3.4 Å. Previous Ca EXAFS studies of native PSII (Cinco *et al.* 2002), Sr EXAFS of Sr-substituted PSII (Cinco *et al.* 1998), and Mn EXAFS (Latimer *et al.* 1995) of Ca-depleted PSII (Latimer *et al.* 1998) have shown the contribution of both Mn–Mn (approx. 3.2 Å) and Mn–Ca (approx. 3.4 Å) vectors to FT peak III. Peak IIIA can be assigned to the Mn–Ca vector and peak IIIB to the Mn–Mn vector, and moreover, the dichroic behaviour of peak IIIB is strikingly similar to that reported previously for the Mn–Sr vector (Cinco *et al.* 2004).

We have extended these studies to the S₀ and S₃ intermediate states of the enzymatic cycle, and the preliminary results show that there is a significant change in the structure of the Mn complex in the S₃ state. This result has important implications for choosing between the many mechanisms that have been proposed for the water oxidation reaction. In conjunction with the data

from single crystal XAS studies, these studies promise to reveal the changes that occur in the structure of Mn₄Ca catalyst as it cycles through the S-states and the mechanism of water oxidation.

3. ELECTRONIC STRUCTURE OF THE Mn₄Ca CLUSTER

(a) X-ray resonant Raman or resonant inelastic X-ray scattering

A key question for the understanding of photosynthetic water oxidation is whether the four oxidizing equivalents generated by the reaction centre are accumulated on the four Mn ions of the OEC during S-state turnover, or whether a ligand-centred oxidation takes place, especially, before the formation and release of molecular oxygen during the S₃ to (S₄) to S₀ transition (Yano & Yachandra 2007). It is crucial to solve this problem, because the Mn redox states form the basis for any mechanistic proposal. The description of the Mn₄Ca cluster in the various S-states in terms of the formal oxidation states is very useful, and Mn K-edge XANES has been the conventional X-ray spectroscopic method for determining the oxidation states. However, it is also important to understand the electronic structure of the Mn cluster in more detail, and we are using the technique of X-ray resonant Raman or resonant inelastic X-ray scattering (RIXS) that has potential for addressing this issue. The RIXS data are collected by scanning the incident energy (1s to 3d/4p absorption followed by 2p-1s emission) to yield two-dimensional plots that can be interpreted along the incident energy axis or the energy transfer axis, which is the difference between the incident and emission energies (figure 4*a*; Glatzel & Bergmann 2005). This method provides a description of the degree of covalency and d-orbital occupancy of the Mn complex, both parameters that are critical for understanding what makes this cluster function as it advances through the enzymatic cycle.

We have compared the spectral changes in the RIXS spectra between the Mn₄Ca cluster in the S-states (Glatzel *et al.* 2004), Mn oxides (Glatzel *et al.* 2005) and Mn coordination complexes, and the results indicate clear differences in how the protein environment modulates the Mn atoms, leading to stronger covalency for the electronic configuration in the Mn₄Ca cluster compared with that in the oxides or coordination complexes.

The RIXS two-dimensional data are best shown as contour plots (figure 4*b*). The comparison of Mn(II), Mn(III), Mn(IV) and PSII in the S₁ and S₂ states shows that both states contain a mixture of oxidation states Mn(III) and Mn(IV). The integrated cross sections along the Raman or energy transfer axis are the L₃-like edge (2p to 3d; figure 4*c*, (i)). The integrated cross sections along the incident energy are the K-edge (1s to 3d) transition (figure 4*c*, (ii)). It is clear from figure 4 that Mn in the S₁ state is in oxidation states III and IV, thus providing confirmation for the (III₂, IV₂) assignment for the S₁ state.

The first moment of the spectrum integrated along the incident energy axis (see figure 4*b*) was calculated for all the S-states and compared with the first moments obtained from Mn oxides and Mn

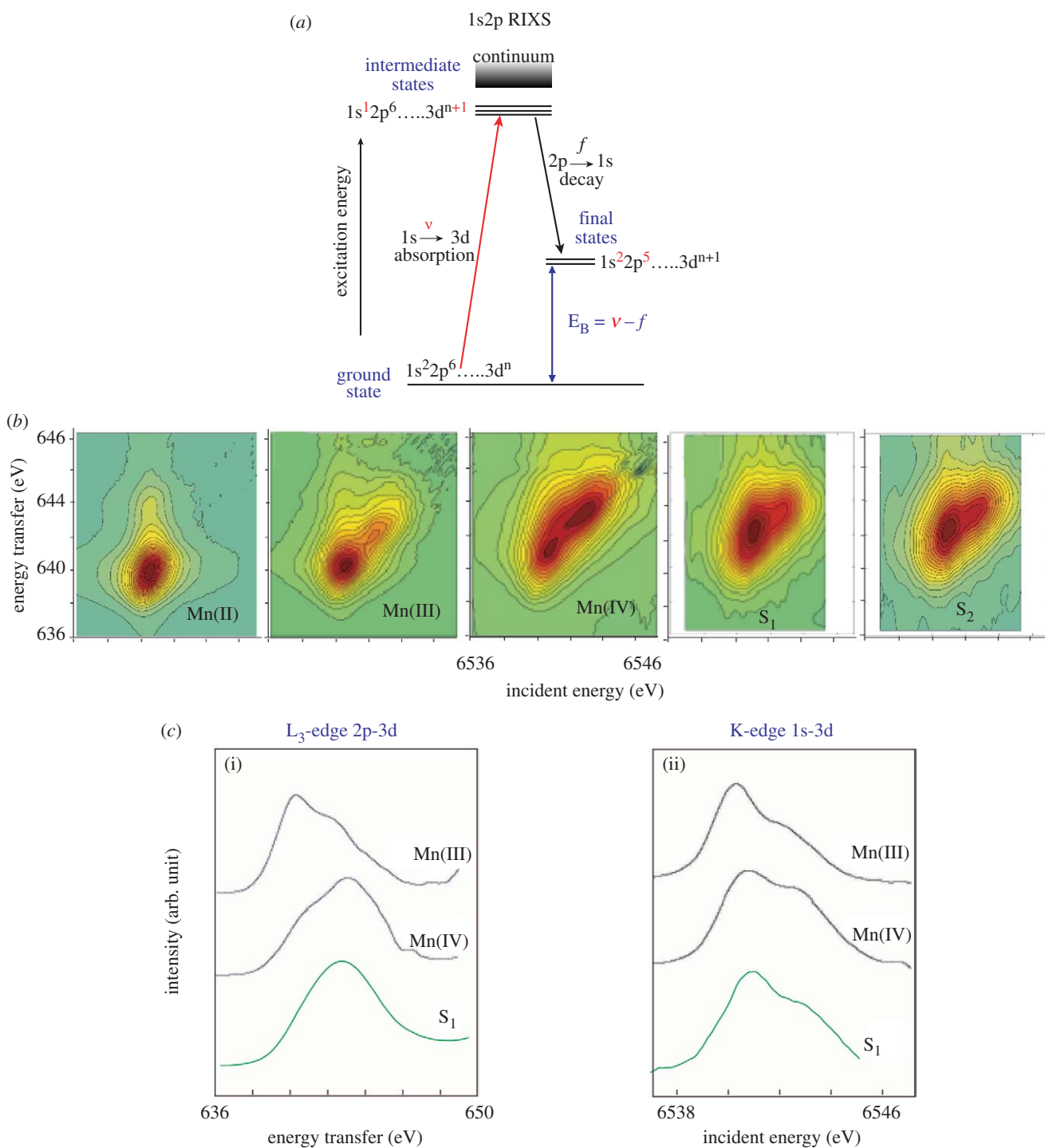


Figure 4. (a) The energy level diagram for 1s2p RIXS. Excitation energy ν , corresponding to a 1s to 3d transition, is scanned using a double crystal monochromator; f is the 2p to 1s emission that is scanned using a high-resolution crystal monochromator. The difference $\nu - f$ corresponds to an L-edge-like (2p to 3d) transition. (b) Contour plots of the 1s2p_{3/2} RIXS planes for three molecular complexes $Mn^{II}(acac)_2(H_2O)_2$, $Mn^{III}(acac)_3$, and $Mn^{IV}(sal)_2(bipy)$ and PSII in the S₁- and S₂ state. One axis is the excitation energy and the other is the energy transfer axis. The L-edge like spectra are along the energy transfer axis and the 1s to 3d transition is along the incident energy axis. The assignment of Mn(III₂,IV₂) for the S₁ state is confirmed by these spectra. (c(ii)) Integrated spectra along the incident energy axis (K-edge, 1s to 3d) and (c(i)) energy transfer axis (L₃ edge, 2p to 3d) of the contour plot shown in figure 4b for Mn^{III}(acac)₃ and Mn^{IV}(sal)₂(bipy) and S₁ state of PSII. The spectra show that the Mn oxidation state of S₁ is a combination of (III) and (IV).

coordination compounds in formal oxidation states of (II), (III) and (IV).

The changes per oxidation state in the first moment positions are more pronounced between the Mn oxides than between the Mn coordination complexes. This observation is consistent with a stronger covalency in the coordination complexes. We furthermore observe a

Mn oxidation between the S₁ and S₂ states of PSII, even though the change in the Mn electronic structure is less pronounced than in the oxides. The spectral change per Mn ion between Mn^{III}(acac)₃ and Mn^{IV}(salicylate) (bipy) is by a factor of 2 more pronounced than between S₁ and S₂. In other words, the orbital population change Δn_{3d}^{eff} per change in

oxidation state is largest between the Mn oxides and smallest between S₁ and S₂. The reason is an increased covalency or delocalization of the Mn valence orbitals in the Mn₄Ca cluster in PSII. We thus find that the electron that is transferred from the OEC in PSII between S₁ and S₂ is strongly delocalized.

Although there has been general agreement with respect to the increasing oxidation of Mn in the cluster during S₀ to S₁ and S₁ to S₂, there is a lack of consensus concerning Mn oxidation during S₂ to S₃. It is also not clear whether the S₀ state contains any Mn(II). Recent preliminary results from the S₀ and S₃ states show that the orbital population change $\Delta n_{3d}^{\text{eff}}$ per change in oxidation state between the S₂ and S₃ states is half as much as that between S₀ and S₁, or S₁ and S₂. These results indicate that the electron is removed from a more covalent form or a more delocalized orbital during the S₂ to S₃ transition compared with the S₀ to S₁ and S₁ to S₂ transitions.

These results are in agreement with the earlier qualitative conclusions derived from the shifts in the XANES inflection point energies and the K β emission peaks from the S-states and support considering a predominantly ligand-based oxidation during the S₂ to S₃ transition (Messinger *et al.* 2001; Yano & Yachandra 2007).

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REFERENCES

- Bergmann, U. & Cramer, S. P. 1998 A high-resolution large-acceptance analyzer for X-ray fluorescence and Raman spectroscopy. In *SPIE Conference on Crystal and Multilayer Optics*, vol. 3448, pp. 198–209. San Diego, CA: SPIE.
- Cinco, R. M., Robblee, J. H., Rompel, A., Fernandez, C., Yachandra, V. K., Sauer, K. & Klein, M. P. 1998 Strontium EXAFS reveals the proximity of calcium to the manganese cluster of oxygen-evolving Photosystem II. *J. Phys. Chem. B* **102**, 8248–8256. (doi:10.1021/jp981658q)
- Cinco, R. M., Holman, K. L. M., Robblee, J. H., Yano, J., Pizarro, S. A., Bellacchio, E., Sauer, K. & Yachandra, V. K. 2002 Calcium EXAFS establishes the Mn–Ca cluster in the oxygen-evolving complex of photosystem II. *Biochemistry* **41**, 12 928–12 933. (doi:10.1021/bi026569p)
- Cinco, R. M., Robblee, J. H., Messinger, J., Fernandez, C., Holman, K. L. M., Sauer, K. & Yachandra, V. K. 2004 Orientation of calcium in the Mn₄Ca cluster of the oxygen-evolving complex determined using polarized strontium EXAFS of Photosystem II membranes. *Biochemistry* **43**, 13 271–13 282. (doi:10.1021/bi036308v)
- Cramer, S. P. 1988 Biochemical applications of X-ray absorption spectroscopy. In *X-ray absorption: principles, applications and techniques of EXAFS, SEXAFS, and XANES* (eds D. C. Koningsberger & R. Prins), pp. 257–326. New York, NY: Wiley-Interscience.
- Dau, H., Andrews, J. C., Roelofs, T. A., Latimer, M. J., Liang, W., Yachandra, V. K., Sauer, K. & Klein, M. P. 1995 Structural consequences of ammonia binding to the manganese cluster of the photosynthetic oxygen-evolving complex: an X-ray absorption study of isotropic and oriented photosystem II particles. *Biochemistry* **34**, 5274–5287. (doi:10.1021/bi00015a043)
- Dau, H., Iuzzolino, L. & Dittmer, J. 2001 The tetra-manganese complex of photosystem II during its redox cycle—X-ray absorption results and mechanistic implications. *Biochim. Biophys. Acta* **1503**, 24–39. (doi:10.1016/S0005-2728(00)00230-9)
- Ferreira, K. N., Iverson, T. M., Maghlaoui, K., Barber, J. & Iwata, S. 2004 Architecture of the photosynthetic oxygen-evolving center. *Science* **303**, 1831–1838. (doi:10.1126/science.1093087)
- Flank, A. M., Weininger, M., Mortenson, L. E. & Cramer, S. P. 1986 Single-crystal EXAFS of nitrogenase. *J. Am. Chem. Soc.* **108**, 1049. (doi:10.1021/ja00265a034)
- Garman, E. 2003 Cool crystals: macromolecular cryocrystallography and radiation damage. *Curr. Opin. Struct. Biol.* **13**, 545–551. (doi:10.1016/j.sbi.2003.09.013)
- George, G. N., Prince, R. C. & Cramer, S. P. 1989 The manganese site of the photosynthetic water-splitting enzyme. *Science* **243**, 789–791. (doi:10.1126/science.2916124)
- Glatzel, P. & Bergmann, U. 2005 High resolution 1s core hole X-ray spectroscopy in 3d transition metal complexes—electronic and structural information. *Coord. Chem. Rev.* **249**, 65–95. (doi:10.1016/j.ccr.2004.04.011)
- Glatzel, P. *et al.* 2004 The electronic structure of Mn in oxides, coordination complexes, and the oxygen-evolving complex of photosystem II studied by resonant inelastic X-ray scattering. *J. Am. Chem. Soc.* **126**, 9946–9959. (doi:10.1021/ja038579z)
- Glatzel, P., Yano, J., Bergmann, U., Visser, H., Robblee, J. H., Gu, W., de Groot, F. M. F., Cramer, S. P. & Yachandra, V. K. 2005 Resonant inelastic X-ray scattering (RIXS) spectroscopy at the Mn K absorption pre-edge—a direct probe of the 3d orbitals. *J. Phys. Chem. Solid.* **66**, 2163–2167. (doi:10.1016/j.jpics.2005.09.012)
- Grabolle, M., Haumann, M., Müller, C., Liebisch, P. & Dau, H. 2006 Rapid loss of structural motifs in the manganese complex of oxygenic photosynthesis by X-ray irradiation at 10–300 K. *J. Biol. Chem.* **281**, 4580–4588. (doi:10.1074/jbc.M509724200)
- Henderson, R. 1990 Cryo-protection of protein crystals against radiation damage in electron and X-ray diffraction. *Proc. R. Soc. B* **241**, 6–8. (doi:10.1098/rspb.1990.0057)
- Kamiya, N. & Shen, J. R. 2003 Crystal structure of oxygen-evolving photosystem II from *Thermosynechococcus vulcanus* at 3.7-angstrom resolution. *Proc. Natl Acad. Sci. USA* **100**, 98–103. (doi:10.1073/pnas.0135651100)
- Kok, B., Forbush, B. & McGloin, M. 1970 Cooperation of charges in photosynthetic O₂ evolution. *Photochem. Photobiol.* **11**, 457–476.
- Latimer, M. J., DeRose, V. J., Mukerji, I., Yachandra, V. K., Sauer, K. & Klein, M. P. 1995 Evidence for the proximity of calcium to the manganese cluster of photosystem II: determination by X-ray absorption spectroscopy. *Biochemistry* **34**, 10 898–10 909. (doi:10.1021/bi00034a024)
- Latimer, M. J., DeRose, V. J., Yachandra, V. K., Sauer, K. & Klein, M. P. 1998 Structural effects of calcium depletion on the manganese cluster of photosystem II: determination by X-ray absorption spectroscopy. *J. Phys. Chem. B* **102**, 8257–8265. (doi:10.1021/jp981668r)
- Loll, B., Kern, J., Saenger, W., Zouni, A. & Biesiadka, J. 2005 Towards complete cofactor arrangement in the 3.0 Å

- resolution structure of photosystem II. *Nature* **438**, 1040–1044. (doi:10.1038/nature04224)
- Messinger, J. et al. 2001 Absence of Mn-centered oxidation in the S₂ to S₃ transition: implications for the mechanism of photosynthetic water oxidation. *J. Am. Chem. Soc.* **123**, 7804–7820. (doi:10.1021/ja004307+)
- Mukerji, I., Andrews, J. C., Deroose, V. J., Latimer, M. J., Yachandra, V. K., Sauer, K. & Klein, M. P. 1994 Orientation of the oxygen-evolving manganese complex in a photosystem-II membrane preparation—an X-ray-absorption spectroscopy study. *Biochemistry* **33**, 9712–9721. (doi:10.1021/bi00198a042)
- Penner-Hahn, J. E. 1998 Structural characterization of the Mn site in the photosynthetic oxygen-evolving complex. *Struct. Bond.* **90**, 1–36.
- Pushkar, Y., Yano, J., Glatzel, P., Messinger, J., Lewis, A., Sauer, K., Bergmann, U. & Yachandra, V. K. 2007 Structure and orientation of the Mn₄Ca cluster in plant photosystem II membranes studied by polarized range-extended X-ray absorption spectroscopy. *J. Biol. Chem.* **282**, 7198–7208. (doi:10.1074/jbc.M610505200)
- Rutherford, A. W. 1985 Orientation of EPR signals arising from components in photosystem II membranes. *Biochim. Biophys. Acta* **807**, 189–201. (doi:10.1016/0005-2728(85)90122-7)
- Sauer, K., Yano, J. & Yachandra, V. K. 2008 X-ray spectroscopy of the photosynthetic oxygen-evolving complex. *Coord. Chem. Rev.* **252**, 318–335. (doi:10.1016/j.ccr.2007.08.009)
- Scott, R. A. 1984 X-ray absorption spectroscopy. In *Structural and resonance techniques in biological research* (ed. D. L. Rousseau), pp. 295–362. Orlando, FL: Academic Press.
- Scott, R. A., Hahn, J. E., Doniach, S., Freeman, H. C. & Hodgson, K. O. 1982 Polarized X-ray absorption spectra of oriented plastocyanin single crystals. Investigation of methionine–copper coordination. *J. Am. Chem. Soc.* **104**, 5364–5369. (doi:10.1021/ja00384a020)
- Wydrzynski, T. & Satoh, S. 2005 *Photosystem II: the light-driven water: plastoquinone oxidoreductase. Advances in photosynthesis and respiration*. Dordrecht, The Netherlands: Springer.
- Yachandra, V. K. 1995 X-ray absorption spectroscopy and applications in structural biology. *Methods Enzymol.* **246**, 638–675.
- Yachandra, V. K. 2005 The catalytic manganese-cluster: organization of the metal ions. In *Photosystem II: the light-driven water: plastoquinone oxidoreductase* (eds T. Wydrzynski & S. Satoh), pp. 235–260. Dordrecht, The Netherlands: Springer.
- Yano, J. & Yachandra, V. K. 2007 Oxidation state changes of the Mn₄Ca cluster in photosystem II. *Photosyn. Res.* **92**, 289–303. (doi:10.1007/s11120-007-9153-5)
- Yano, J. et al. 2005a X-ray damage to the Mn₄Ca complex in photosystem II crystals: a case study for metallo-protein X-ray crystallography. *Proc. Natl Acad. Sci. USA* **102**, 12 047–12 052. (doi:10.1073/pnas.0505207102)
- Yano, J., Pushkar, Y., Glatzel, P., Lewis, A., Sauer, K., Messinger, J., Bergmann, U. & Yachandra, V. K. 2005b High-resolution Mn EXAFS of the oxygen-evolving complex in photosystem II: structural implications for the Mn₄Ca cluster. *J. Am. Chem. Soc.* **127**, 14 974–14 975. (doi:10.1021/ja054873a)
- Yano, J. et al. 2006 Where water is oxidized to dioxygen: structure of the photosynthetic Mn₄Ca cluster. *Science* **314**, 821–825. (doi:10.1126/science.1128186)
- Zouni, A., Witt, H.-T., Kern, J., Fromme, P., Krauß, N., Saenger, W. & Orth, P. 2001 Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution. *Nature* **409**, 739–743. (doi:10.1038/35055589)

Discussion

C. Dismukes (*Princeton University, USA*). Do your RIXS data provide more proof for the oxidation state assignments for the Mn ions in PSII versus the earlier XANES data that rely entirely on comparison with reference model compounds?

V. Yachandra. RIXS spectra provide a way for obtaining the effective number of electrons on Mn and also the degree of covalency. Therefore one can reliably determine the charge on the metal, which is Mn₄(III₂,IV₂) in the S₁ state, and the changes between the S-states can tell us about the difference in the charge and in the degree of covalency. We find that the electron that is extracted from the OEC between S₁ and S₂ is strongly delocalized, consistent with strong covalency for the electronic configuration in the OEC. The orbital population change per change in oxidation state between the S₂ and S₃ states is half as much as that between that between S₀ and S₁, or S₁ and S₂ transitions, indicating that the electron is removed from a more covalent form or even more delocalized orbital during this transition.

R. Pace (*Australian National University, Canberra, Australia*). Is the overall dichroism seen in the EXAFS pattern greater in the oriented PSII membranes than in the rotation patterns from single crystal? V. Yachandra. The observed dichroism is different for the oriented PSII membranes and crystals, as expected. PSII membranes are oriented in the *xy* plane, and hence there is no discrimination between *x* and *y*, or *+z* and *-z* (top and bottom of the PSII membrane). On the other hand, PSII crystals are organized in three dimensions containing four molecules (PSII membranes)/unit cell, which contribute to the differences compared with oriented PSII membranes, and also generate the complicated dichroism pattern. The other parameter that influences the dichroism is the mosaic spread, which is very small (approx. 0.1 degrees) for crystals, but can be large (approx. 20 degrees) for oriented PSII membranes.

J. Barber (*Imperial College London, UK*). Although EXAFS can give high-resolution spatial information for the Mn cluster, is it correct to call your overall model of the OEC including Ca²⁺ and the amino acid residues ‘high resolution’? Surely the quality of model is dependent on the accuracy of the positioning of the Ca²⁺ and the amino acid side chains which at present are at intermediate resolution and perhaps modified by radiation damage.

V. Yachandra. The structure of the Mn cluster determined from single crystal EXAFS is at an accuracy of approximately 0.02 Å in distances and approximately 0.15 Å in resolution. Moreover, the structure of the Mn cluster is independent of the details of the PSII structure determined from X-ray diffraction, or the positioning of the amino acid side chains. Therefore, we think it is justified to refer to the EXAFS-derived Mn cluster as a high-resolution structure, as we are referring only to the Mn cluster, and it does not have anything to do with the overall PSII structure or the vicinity of the Mn cluster in the OEC.