An e.x.a.f.s. study of the manganese O₂-evolving complex in purified Photosystem II membrane fractions

The S₁ and S₂ states

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Manganese K-edge X-ray spectra have been obtained for Photosystem II samples isolated using Triton X-100 detergent and samples further purified by n-heptyl β -D-thioglucoside detergent treatment to remove light-harvesting polypeptides and low-affinity calcium. The structure of the manganese complex is very similar for the two detergent preparations used. Analysis of the e.x.a.f.s. spectra for samples in the S₁ and S₂ states indicate changes in bond lengths for the shells of oxygen/nitrogen atoms. For the S₁ state, oxygen shells at 0.181 and 0.193 nm (1.81 and 1.93 Å) were observed and one manganese shell at 0.270 nm (2.70 Å). In the S₂ state the oxygen bond lengths are longer at 0.184 and 0.200 nm (1.84 and 2.00 Å). Additionally a shell of scatterers at 0.37 nm (3.7 Å) was observed in both states which could be fitted to models with calcium scatterers at this distance.

INTRODUCTION

Photosynthetic reaction centres catalyse the conversion of light into chemical energy. In plants and cyanobacteria, part of this reaction involves the photosynthetic oxidation of water in a four-electron transfer process by a membrane-bound chlorophyll-protein complex, photosystem II (PSII) [1]. This oxidation is not well understood, and is thought to involve, as the active centre, a manganese complex. Elemental analysis indicates that four manganese atoms are required per reaction centre [2], and activation of the enzyme also requires the cofactors Ca²⁺, and Cl⁻ [3,4].

In order to accomplish water-oxidation the enzyme cycles through five intermediate states, S_n (n = 0, ..., 4), which were thought to reflect different oxidation states of the metal complex [5,6]. Dark-adapted chloroplasts relax predominantly to the S₁ state [7]. Oxygen is evolved as S₄ is spontaneously converted into S₀ [6].

A multitude of spectroscopic techniques have been applied to the study of the manganese complex. The manganese complex has characteristic e.p.r. signals in the S₂ state; a 'multiline' signal centred on $g = 2$ ($S' = 1/2$) comprising 16–19 lines [8] and a signal at $g = 4.1$ ($S' = 3/2$) [9]. The $g = 2$ signal has been interpreted as being consistent with two dimers, a trimer or tetramer of manganese atoms in the complex [10,11]. Evidence for an intimate relation between water oxidation and the manganese complex has come from broadening of the 'multiline' by H₂¹⁷O [11], narrowing by ²H₂O exchange [13].

X-ray-absorption studies have provided structural information on the manganese complex. Analysis of e.x.a.f.s. spectra can yield bond lengths ($\pm 1\%$) co-ordination numbers ($\pm 10\%$) and the approximate atomic number, Z, of the scatterer. Comparison of K-edge energies with those of model compounds has indicated that states S₁ and S₂ contain predominantly Mn(III) and Mn(IV) [14–20]. Recent evidence from X-ray-absorption spectroscopy [20] and n.m.r. relaxation studies [21] suggest that no oxidation of manganese occurs on the S₂ → S₃ transition. Klein and co-

workers [14–20,22–25] have made extensive e.x.a.f.s. studies on the S₁ and S₂ states of PSII prepared from spinach (*Spinacia oleracea*) at manganese concentrations of 500–800 μ M. These studies concluded that the average manganese co-ordination environment was the same for both states, despite the change in oxidation of the manganese and contained a short Mn–O/N distance at 0.175 nm (1.75 Å), a Mn–O/N shell at ≈ 0.2 nm (≈ 2.0 Å) and at least one Mn scatterer at 0.27 nm (2.70 Å) [19,20]. Corrie *et al.* [26] found similar results for the S₁ state. The Mn–Mn distance and short Mn–O/N distance have been used as evidence for di- μ -oxo-bridged manganese pairs as structural elements in the manganese complex [19,20].

This picture has been questioned by George *et al.* [27] and subsequently Penner-Hahn *et al.* [28], who have obtained S₁ data on more concentrated samples (1.5 mM-Mn for the latter study). Both groups confirm the presence of an Mn–Mn scatterer at 0.27 nm (2.70 Å), but find no evidence for a shell of low-Z scatterers at 0.175–0.185 nm (1.75–1.85 Å). Additionally both groups report the existence of further shells at longer distances. George *et al.* [27], using oriented chloroplasts, resolved a second Mn–Mn shell at 0.33 nm (3.3 Å) whose vector is oriented perpendicular to the membrane plane, and Penner-Hahn *et al.* [28] report a Mn or Ca shell at 0.33 nm (3.3 Å) as well as a possible shell of scatterers at 0.42 nm (4.2 Å). The presence of the 0.33 nm (3.3 Å) Mn–Mn shell has been used as evidence for tri- and tetranuclear manganese complexes as the active site.

The presence of these additional shells of scatterers, and their origin, have important implications for the elucidation of the mechanism of water oxidation and the structure of the manganese complex. This is especially true with regard to the roles in the mechanism of the inorganic cofactors Cl⁻ and Ca²⁺ which may be located near the manganese complex.

Recently we have optimized a procedure for isolating highly concentrated reaction centres using the detergent n-heptyl β -D-thioglucoside (HTG) [29,30]. The reduced amounts of accessory chlorophyll-binding proteins has allowed preparation of samples with 1–3 mM-Mn, permitting an improvement in the quality of

Abbreviations used: PSII, Photosystem II; x.a.n.e.s., X-ray absorption near edge structure; e.x.a.f.s., extended X-ray absorption fine structure; HTG, n-heptyl β -D-thioglucoside; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; BBY, PSII membranes prepared by the method based on that given in [31].

the e.x.a.f.s. data and collection time. Preliminary studies on HTG-treated particles showed the manganese cluster to be susceptible to disproportionation, indicated by the presence of Mn^{2+} , and to radiation damage. By altering the reaction conditions we have prepared highly purified PSII particles for which we do not detect Mn^{2+} or radiation damage after 10 h of X-ray-beam exposure. We have made e.x.a.f.s. measurements at the Mn K-edge on samples poised in both the S_1 and S_2 states prepared from isolated [PSII membranes (BBY)] and highly purified (HTG) membranes. Specifically we wish to clarify the existence of any changes in bond length or co-ordination between the S_1 and S_2 states, the existence of low- Z shell of scatterers at 0.175–0.185 nm (1.75–1.85 Å), as well as the newly proposed shells at 0.33 and 0.43 nm (3.3 and 4.3 Å).

EXPERIMENTAL

Oxygen-evolving PSII subchloroplast membranes were prepared by the method of Berthold *et al.* [31] using Triton X-100 detergent with the modifications described by Ford & Evans [32]; these are termed BBY particles.

Further purification was based on the method of Enami *et al.* [30] with slight modification [29]; membranes thus purified are termed HTG particles. Isolated BBY particles were resuspended to 2 mg of chlorophyll/ml with 1.8% (w/v) HTG/1.0 M-sucrose/40 mM-Mes/NaOH (pH 6.0)/40 mM-MgCl₂/10 mM-NaCl. After incubation on ice for 10 min the mixture was diluted with 1.5 vol. of a solution containing 0.5 M-sucrose, 40 mM-Mes/NaOH, pH 6.0, and centrifuged at 40000 *g* for 45 min. The supernatant was mixed with 2 vol. of 40 mM-Mes/NaOH, pH 6.0, and centrifuged at 40000 *g* for 60 min. The pellet was resuspended in 40 mM-Mes/NaOH (pH 6.0)/10 mM-NaCl/1 mM-EDTA and centrifuged for 10 min at 40000 *g*. The final pellet was resuspended in Buffer A (40 mM-Mes/NaOH (pH 6.0)/10 mM-NaCl/5 mM-MgCl₂/20% glycerol) and stored at 77 K. In these preparations the low-affinity calcium site is thought to be absent.

Samples for e.x.a.f.s. were prepared by first washing the PSII particles in a buffer containing 40 mM-Mes/NaOH (pH 6.0), 10 mM-NaCl and 1 mM-EDTA, followed by centrifugation at 40000 *g* for 10 min. Samples for loading into the e.x.a.f.s. holders were then prepared by resuspension in Buffer A, but containing only 10% glycerol, and pelleting the membranes at 120000 *g* for 1 h. No adventitious Mn^{2+} was detected by e.p.r. at 8 K using a JEOL RE1X spectrometer with an Oxford Instruments liquid-helium cryostat.

S_1 samples were prepared by first exposing the samples to a short period of illumination to randomize the S_n states, followed by a 4 h dark adaption. A BBY S_2 sample was prepared by 200 K illumination of a dark-adapted S_1 sample for 30 min. For the HTG S_2 samples, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added to limit the reaction centres to a single turnover and then the samples were frozen at 77 K under illumination after 30 sec illumination at 277 K. The samples were estimated to be 500–800 μM -Mn for BBY preparations and 1–2 mM-Mn for HTG preparations.

Fluorescence e.x.a.f.s. [33] measurements were carried out at the Synchrotron Radiation Source at the SERC Daresbury Laboratory, using station 8.1 with a slitless Si(III) double-crystal monochromator and a thirteen-element Ge solid-state detector [34]. Spectra were recorded at 77 K with a beam energy of 2 GeV and an average beam current of 160 mA. Each scan lasted approx. 1 h. For the BBY preparations, ten scans were averaged, whereas for the HTG preparations five to ten scans were used per sample. Data from each of the detector elements

were examined separately for anomalies and weighted by the edge-jump before averaging and energy calibration with the Daresbury EXCALIB program [35]. The averaged spectra were normalized to a unit metal atom, and the e.x.a.f.s. were extracted by removing the background absorption using the Daresbury EXBACK program [35]. The e.x.a.f.s. were weighted by k^3 and analysed by using the non-linear least-squares minimization program EXCURV90 [36], which calculates the theoretical e.x.a.f.s. using the fast-curved-wave approach [37]. Previous studies have utilized the plane-wave approximation [38] for the scattering of the photoelectron wave by neighbouring atoms. Phase shifts were calculated by 'ab initio' methods within the EXCURV90 program [39] and verified with model compounds [40–42]. Although it is usual to approximate the excited central atom with wave functions derived from a $Z+1$ atom with one electron from the 1 s orbital removed [43], a better agreement with crystallographic data on the model compounds was achieved using wave functions from the central atom, Mn, with a relaxed 1 s core hole. Differences between crystallographic distances and theory ranged between 0 and -0.004 nm (0 and -0.04 Å) (Table 1). That we are able to fit model compounds with our *ab initio* phase shifts shows that our results are independent of the minor phase and amplitude differences expected for Mn(III) and Mn(IV) oxidation states. Analysis of model compounds gave an amplitude reduction factor for multielectron absorption of 0.7, whereas the imaginary part of the photoelectron self-energy was set to -1 eV in order to reproduce the elastic electron mean free path. The photoelectron energy zero, E_0 , was treated as a single overall parameter in the multiple-shell fits. E_0 refined to values close to those obtained for model compounds, 20–30 eV, below the experimental absorption threshold. The Fourier transforms were calculated with the plane-wave approximation and corrected for the phase shift of the first shell of back-scattering atoms, resulting in peaks at approximately the expected radial distance [44]. Data analysis was performed on the raw e.x.a.f.s. in k space with use of the Fourier transform confined to model building and visual determination of quality of fit. Structural models were compared by comparing the fit index, FI (N. Binsted, R. W. Strange & S. S. Hasnain, unpublished work), defined as:

$$\Sigma_i^N 1/\sigma_i^2 [\chi^{\text{exp}}(k_i) - \chi^{\text{the}}(k_i)]^2$$

where $1/\sigma_i = k_i^n / \Sigma_j^N k_j^n |\chi^{\text{exp}}(k_j)|$, N is the number of data points, n is selected to produce a constant envelope amplitude for $k^n \chi^{\text{exp}}(k)$ ($n = 3$ for k^3 weighted data) the sum of which is the experimental amplitude of the spectra and is a constant, and the agreement factor, R , defined as:

$$\Sigma_i^N 1/\sigma_i^2 [|\chi^{\text{exp}}(k_i) - \chi^{\text{the}}(k_i)|] 100 \%$$

Use was also made of an absolute index of goodness-of-fit given by the reduced χ^2 function [45, 46]:

$$\epsilon_r^2 = 1/(N_{\text{ind}} - p)(N_{\text{ind}}/N) \Sigma_i^N 1/\sigma_i^2 [\chi^{\text{exp}}(k_i) - \chi^{\text{the}}(k_i)]^2$$

where N_{ind} is the number of independent (ind) points, $N_{\text{ind}} = 2(r_{\text{max}} - r_{\text{min}})(k_{\text{max}} - k_{\text{min}})/\pi$ and p is the number of parameters.

For fitting purposes the only light atoms co-ordinated to the manganese were considered to be oxygen atoms. This is consistent with current evidence which suggests only a small number of nitrogen ligands [47, 48]. We adopted a fitting procedure whereby the co-ordination numbers were varied systematically in steps of 1.0 while the Debye–Waller factors, $2\sigma^2$, where σ is the root-mean-square deviation in the inter-atomic distance, were refined to obtain the best simulation. The Debye–Waller factors reflect the thermal and static disorder in the bond distances. Co-ordination numbers and Debye–Waller factors were refined together using different initial values for these parameters. From

Table 1. Curve-fitting results for model compounds (k^3 -weighted e.x.a.f.s.)

Compound (Cpd.) co-ordination numbers and shell sequences from crystal structure were as follows: 1, α -MnO₂, 6(O) 2(Mn) 8(Mn), [40]; 2, Mn(III) Mn(IV) (μ_2 -O)₂(bipyridyl)₄(ClO₄)₃, 2(O) 4(N) 1(Mn) 8(C) [41]; 3, Mn(III)₄(μ_3 -O)₂(OAc)₇(bipyridyl)₂(ClO₄)₃, 1.5(O) 4.5(O) 0.5(Mn) 2(Mn) [42]. Values in parentheses are averages calculated from the crystal structure of each model; r_n are scatterer absorber distances in Å (1 Å = 0.1 nm). The Debye-Waller factors $\alpha n = 10^2$ $2\sigma^2$ Å² (σ = root-mean-square deviation in inter-atomic distance).

Parameter	Cpd...	1	2	3
r_1		1.88 (1.89)	1.78 (1.82)	1.89 (1.88)
r_2		2.86 (2.87)	2.07 (2.11)	2.07 (2.07)
r_3		3.42 (3.45)	2.71 (2.72)	2.84 (2.85)
r_4		—	2.98 (3.02)	3.32 (3.34)
α_1		0.8	1.7	0.2
α_2		0.7	3.2	3.8
α_3		0.8	0.6	0.5
α_4		—	1.8	1.3
E_0		25.2	26.4	20.0
N_{ind}		15.4	20.6	15.7
p		7	9	9
$10^3 \times \text{FI}$		0.91	1.04	0.65
R		27.5	39.7	24.3
$10^6 \times \epsilon_v^2$		13.3	17.9	7.2

these calculations the errors associated with the co-ordination numbers are $\pm 20\%$ for the short oxygen and manganese shells and ± 30 – 50% for the longer oxygen shell. The estimated error for the radii are $\leq 2\%$ and, for the Debye-Waller factors, ± 30 – 50% .

RESULTS

X.a.n.e.s.

Mn K-edge spectra of samples poised in the S_1 and S_2 states appear to be identical for BBY and HTG samples. No changes were caused by the further purification of the BBY particles to produce HTG-treated particles. However, there are small differences between the S_1 and S_2 samples. On going from S_1 to S_2 states the edge position is shifted ≈ 1.1 eV from 6551.4 eV (Mn foil, edge 6539 eV) to higher energies, indicating an increase in manganese oxidation state, confirming the observations of Klein, Sauer and co-workers [24,25].

E.x.a.f.s.

Figs. 1 and 2 show the raw k^3 -weighted e.x.a.f.s. data and Fourier transforms obtained for BBY (ten scans) and HTG particles in both the S_1 (two scans) and S_2 (five scans) states. It is apparent from Fig. 1 that the e.x.a.f.s. modulations and the Fourier transforms of the two preparations show strong similarities, except for the difference in the intensity of the Fourier-transform peak at ≈ 0.37 nm (≈ 3.7 Å). The amplitudes of the e.x.a.f.s. oscillations for the S_1 and S_2 states differ slightly. The BBY S_2 spectrum is not shown, as this sample had a reduced signal-to-noise ratio; however, the spectrum was very similar to that of the HTG S_2 sample.

The Fourier transforms of the k^3 data reveal three peaks (Fig. 2) corresponding to shells of scatterers at ~ 0.2 , 0.27 and 0.37 nm (2, 2.7 and 3.7 Å). Although there are possibly features in the Fourier transform at > 0.4 nm (> 4 Å) in the HTG S_1 sample, we are unable to confirm their presence with the current signal-to-noise in the data.

Fig. 1. k^3 -Weighted e.x.a.f.s. spectra of (a) a dark-adapted BBY PSII preparation, S_1 state, (b) a HTG PSII preparation poised in the S_1 state and (c) a HTG PSII preparation poised in the S_2 state

The continuous line represents experimental data and the broken line the theoretical result. For details of the fits, see Table 4.

Simulations of scatterers located by the first Fourier-transform peak

On fitting a single shell of oxygen atoms to the raw data, at the distance indicated by the first Fourier-transform peak, a best-fit distance of 0.184 – 0.187 nm (1.84 – 1.87 Å) and co-ordination number of 4.0 were obtained for all the samples. The presence of additional shells of scatterers at ≈ 0.2 nm (≈ 2.0 Å) was indicated by the poor fit to both the modulations and Fourier transform, as illustrated in the Fourier-filtered BBY S_1 data shown in Fig. 3(a) and the improved fit obtained for the two-shell model (Fig. 3b; Table 2). Inclusion of the additional shell resulted in an $\approx 50\%$ reduction of the fit index, FI, agreement factor, R , and a significant improvement in ϵ_v^2 for the Fourier-filtered shell for all the samples (Table 2). Best-fit Mn–O distances of 0.180 and 0.193 nm (1.80 and 1.93 Å) for the BBY S_1 samples

Fig. 2. k^3 -weighted Fourier transform of the e.x.a.f.s. data for spectra of (a) a dark-adapted BBY PSII preparation, S_1 state, (b) a HTG PSII preparation poised in the S_1 state and (c) a HTG PSII preparation poised in the S_2 state

The continuous line represents the experimental data and the broken line the theoretical result. For details of the fits, see Table 4.

(and equivalent distances for others) are separated by a distance, Δr , close to the resolution limit of the data and thus cannot be expected to be visible in the Fourier-transform peak. However, the least-squares minimization of the k -space data strongly favours a split shell of oxygen atoms, as is clearly demonstrated by Fig. 3 and Table 2. This is not surprising, as neighbour atoms separated by ≈ 0.13 nm (≈ 1.3 Å) are routinely determined to an accuracy of < 0.02 nm (< 0.2 Å) in protein crystallographic determinations where data resolution logically is in excess of 0.2 nm (2.0 Å). We note that the number of independent points for the Fourier-filtered data range from 6.6 to 8.5 and thus the two-shell model with four refinable parameters is significantly overdetermined.

The co-ordination number reflects disorder in the longer oxygen shell, as indicated by the larger Debye–Waller factors for the longer distance, rather than indicating four co-ordinate manganese. For the S_2 samples the best-fit distances for both

Fig. 3. Fourier-filtered k^3 -weighted e.x.a.f.s. data for the first Fourier-transform peak of BBY S_1 state preparation of PSII [distance limits for the Fourier filter, not phase-corrected: 0.09 nm (0.9 Å) $< r <$ 0.2 nm (2.0 Å)]

The continuous line represents the experimental data and the broken line the theoretical result. To aid comparison, E_0 was set to 26.75 eV; (a) best one-shell fit (model 1, Table 3), FI = 0.81×10^{-3} ; and (b) best two-shell fit (model 2, Table 3), FI = 0.36×10^{-3} .

shells were longer by 0.003 and 0.006 nm (0.03 and 0.06 Å), with greater disorder in the second oxygen shell compared with the S_1 samples.

Although Corrie *et al.* [26] preferred to fit three oxygen shells, attempts to fit a third shell of oxygen atoms did not result in a statistically significant improvement in fit quality and is therefore not justified.

The distance for the two oxygen shells has been subject to disagreement in the literature. Recent work by George *et al.* [27] and Penner-Hahn *et al.* [28] had indicated that the 0.18 nm (1.8 Å) distance was not required to fit the S_1 data. However, in all our simulations a short ≤ 0.185 nm (≤ 1.85 Å) Mn–O distance is required [see Tables 2 and 3 and Table 5 (below)]. We were unable to satisfactorily fit the data by using the models described by Penner-Hahn *et al.* [28] or George *et al.* [27]; the Fit Index for both models was more than twice that of the best BBY S_1 simulations in the present study when the distances were fixed at those reported [28,29], and the co-ordination numbers, Debye–Waller factors and E_0 varied. This discrepancy between our results and those of these two reports [28,29] is possibly due to the presence of some Mn^{2+} contamination in the samples described by Penner-Hahn *et al.* [28], resulting in longer Mn–O distance estimates, and the use of partially dehydrated membranes for the data of George *et al.* [27]; Mn(II)–O distances are typically > 0.21 nm (> 2.1 Å) [49,50].

The existence of a short Mn–O distance in the oxygen-evolving complex indicated by the studies of Corrie *et al.* [26] and Klein and collaborators [18–20] is confirmed in our simulations. Our

Table 2. Curve-fitting results for Fourier-filtered data for the first shell of scatterers; one- and two-shell fits to k^3 -weighted e.x.a.f.s. with E_0 set to those of the final fits in Table 4

Parameters are defined in Table 1 and in the text.

Parameter	BBY S ₁		HTG S ₁		BBY S ₂		HTG S ₂	
	4O	2O,2O	4O	2O,2O	4O	2O,2O	4O	2O,2O
r_1	1.86	1.81	1.84	1.80	1.87	1.84	1.86	1.83
r_2	—	1.95	—	1.92	—	1.98	—	2.02
α_1	2.6	0.6	2.4	1.2	2.0	0.6	2.7	0.7
α_2	—	1.4	—	2.0	—	2.5	—	2.9
N_{ind}	6.6	6.6	6.6	6.6	8.5	8.5	7.7	7.7
p	2	4	2	4	2	4	2	4
$10^3 \times \text{FI}$	0.81	0.36	0.28	0.12	1.70	1.11	1.98	0.58
R	30.7	16.6	17.2	10.9	41.1	30.0	49.6	22.6
$10^6 \times \epsilon_p^2$	6.95	5.56	2.37	1.88	13.3	12.4	16.0	7.25

Table 3. Curve-fitting results to the raw data for the BBY S₁ sample; k^3 -weighted e.x.a.f.s. with E_0 set to 26.7 eV

Models have the following co-ordination numbers and shell sequences: 1, 4(O); 2, 2(O) 2(O); 3, 2(O) 2(O) 1(Mn); 4, 2(O) 2(O) 1(Ca); 5, 2(O) 2(O) 1(Mn) 1(Mn); 6, 2(O) 2(O) 1(Mn) 1(Cl). Parameters are defined in Table 1 and in the text; values in parentheses refer to Fourier-filtered data; distance limits for Fourier filter, phase not corrected: 0.07–1.00 nm (0.7–10.0 Å).

Parameter	Model...	1	2	3	4	5	6
r_1		1.85	1.81	1.80	1.80	1.80	1.80
r_2		—	1.93	1.93	1.93	1.93	1.93
r_3		—	—	2.70	2.71	2.70	2.70
r_4		—	—	—	3.66	3.64	3.77
α_1		1.9	0.7	0.7	0.7	0.6	0.7
α_2		—	1.5	1.1	1.2	1.1	1.2
α_3		—	—	0.8	0.7	0.7	0.7
α_4		—	—	—	0.3	0.9	0.3
N_{ind}		17.6	17.6	17.6	17.6	17.6	17.6
p		2	4	6	8	8	8
$10^3 \times \text{FI}$		3.63	3.57	2.70	2.37	2.44	2.44
		(3.53)	(2.78)	(1.75)	(1.00)	(1.13)	(1.21)
R		71.0	69.6	58.1	53.1	54.6	54.7
		(61.9)	(59.7)	(39.5)	(30.1)	(32.5)	(34.5)

results for the two-shell fits are entirely consistent with bond lengths observed in di- μ -oxo-bridged manganese complexes [49–51]. The presence of the disordered shell of terminal low- Z scatterers would be expected for manganese(III), which undergoes Jahn–Teller distortion and/or for a complex containing a mixture of manganese oxidation states [49–51] and could explain the 0.193 nm (1.93 Å) oxygen shell, which is short for terminal ligands. It is possible that the terminal ligands destructively interfere to reveal only some of the ligand distances. This may also explain the change in terminal ligand distance for the S₁ → S₂ transition.

Simulation of the scatterers located by the second Fourier-transform peak

For the distance corresponding to the second Fourier-transform peak a model incorporating 1.0 ± 0.5 Mn at 0.269–0.271 (2.69–2.71 Å) adequately fits the data in agreement with previous studies [19,20,26–28]. Attempts to fit two shells of scatterers at this distance resulted in models with an additional shell of carbon atoms at 0.298 nm (2.98 Å) which was not judged stat-

istically significant. The Mn–Mn distance corresponds well with that observed in di- μ -oxo-bridged manganese complexes [49–51]. That there are 1.0 ± 0.5 Mn–Mn interactions at 0.27 nm (2.7 Å) per manganese suggests there are two or three such interactions present in the four manganese complex, which would indicate that the manganese atoms are minimally arranged as a dimer of μ -oxo-bridged cores, although again we cannot discount larger cores.

Simulation of the scatterers located by the third Fourier-transform peak

A shell of scatterers at > 0.3 nm (> 3 Å) was first proposed by Guiles *et al.* [17] and confirmed by more recent work [27,28]. Manganese was suggested to be the scatterer at 0.33 nm (3.3 Å) [27,28], although Penner-Hahn *et al.* [28] have indicated Ca or the combination of Mn and C. Only three samples had suitable signal-to-noise ratios for the identification of scatterers at > 0.3 nm (> 3 Å): the BBY S₁, HTG S₁ and HTG S₂ samples.

We do not observe scatterers at 0.33 nm (3.3 Å); instead, our samples have scatterers at 0.37 nm (3.7 Å). Some difficulty was experienced in fitting the third Fourier-transform peak with Mn, as might be expected for a polynuclear manganese complex. Inclusion of a shell of scatterers at ≈ 0.37 nm (≈ 3.7 Å) resulted in a ≈ 10 –15% reduction in the fit index for models, including Mn, Ca or Cl, for the raw data for the BBY S₁ (Table 3) HTG S₁ and S₂ samples (results not shown); however, Ca resulted in the best fit.

To illustrate the preference for Ca over other models, the BBY S₁ best three-shell fit for the BBY S₁ sample was subtracted from the data, and the resulting difference Fourier-filtered and modelled (Fig. 4). Fitting of a single shell of Mn scatterers to the feature at 0.37 nm (3.7 Å) does not adequately simulate the modulations (Figs. 4c and 4d). When alternative scatterers are tried, the best fit to the raw data is achieved with Ca for the S₁ BBY sample (for the Fourier-filtered data; fit index 50% lower than for Mn or Cl; ϵ_p^2 also $\approx 50\%$ lower for Ca). In this case only is the phase of the e.x.a.f.s. oscillations and the position of the Fourier-transform peak well produced. For the HTG S₁ and S₂ samples (not shown) the order of preference as determined by fit index was Ca $>$ Mn $\geq$ Cl.

If E_0 is allowed to vary in the Fourier-filtered data, Ca as the scatterer results in the best fit, with an E_0 within a few eV of that for the three-shell fit to the raw data. In the case of Mn or Cl, E_0 changes by several eV compared with the three-shell fit. An analysis of k^2 weighting of the raw data also gave similar results; calcium scatterers at 0.364 nm (3.64 Å) resulted in the best fit to

Fig. 4. Fourier-filtered k^3 -weighted e.x.a.f.s difference spectra for the third Fourier peak of BBY S_1 -state preparation of PSII [distance limits for Fourier filter, 0.27 nm (2.7 Å) $< r < 0.36$ nm (3.6 Å) not phase-corrected]

The continuous line represents experimental data and the broken line the theoretical result; to aid comparison, E_0 was set to 26.75 eV, $N_{\text{ind}} = 3.8$, $p = 2$; (a) and (b) 1Cl, $r = 0.375$ nm (3.75 Å), $\alpha = 0.001$, $\text{FI} = 1.82 \times 10^{-3}$, $R = 50.1$, $\epsilon_v^2 = 19.0 \times 10^{-6}$; (c) and (d) 1Mn, $r = 0.363$ nm (3.63 Å), $\alpha = 0.007$, $\text{FI} = 0.79 \times 10^{-3}$, $R = 30.6$, $\epsilon_v^2 = 8.2 \times 10^{-6}$; and (e) and (f) Ca, $r = 0.366$ nm (3.66 Å), $\alpha = 0.003$, $\text{FI} = 0.39 \times 10^{-3}$, $R = 22.8$, $\epsilon_v^2 = 4.1 \times 10^{-6}$.

the k^2 weighted raw data, with $\epsilon_v^2 \approx 10\%$ better than for Mn or Cl for the BBY S_1 , HTG S_1 and HTG S_2 samples. Thus the primary contribution to this peak is from a scatterer such as Ca.

Although it might be reasonable to assume that the 0.37 nm (3.7 Å) peak results from two or more shells of scatterers, including some carbon atoms from ligands such as tyrosine, the present data does not contain enough information to permit the fitting of additional shells ($N_{\text{ind}} = 3-4$ for the peak of interest).

One reason for the different conclusion reached here compared with earlier work could be the use of glycerol as a cryoprotectant in the present study, as opposed to sucrose in other recent investigations (it is known that the cryoprotectant used can effect the e.p.r. signals observed in the S_2 state). Additionally, the use of the difference method to isolate the third Fourier peak, *ab initio* calculations of e.x.a.f.s. data and improved signal-to-noise ratios obtained for the BBY S_1 and HTG S_2 samples, have enabled us to obtain simulations that favour Ca over Mn as the possible scatterer at 0.37 nm (3.7 Å). For the HTG samples, it is possible that the differences in the intensity of the third Fourier-transform peak compared with the BBY samples are related to the reported reduced Ca content of these preparations (1Ca for HTG; 2Ca for BBY). However, the best-fit models are essentially the same as for the BBY samples, and so it is not possible with the present data to distinguish increased disorder in this shell or a decrease in Ca content. The best fits to the data are presented in Table 4.

Table 5 details the correlation matrix for the BBY S_1 sample

Table 4. Curve-fitting results for all the BBY and HTG samples in the S_1 and S_2 states; k^3 -weighted e.x.a.f.s.

Parameters are defined in Table 1 and the text; values in parentheses refer to Fourier-filtered data; distance limits for Fourier filter, phase not corrected: 0.07–1.00 nm (0.7–10.0 Å).

Parameter	BBY S_1	HTG S_1	BBY S_2	HTG S_2
r_1 (2O)	1.80	1.80	1.85	1.83
r_2 (2O)	1.93	1.92	1.99	2.01
r_3 (1Mn)	2.71	2.69	2.71	2.69
r_4 1(Ca)	3.66	3.69	—	3.67
α_1	0.7	1.4	0.6	0.7
α_2	1.2	2.1	2.8	3.4
α_3	0.7	0.8	1.0	0.8
α_4	0.3	1.4	—	1.2
E_0	26.7	25.1	25.0	25.3
N_{ind}	17.6	13.4	10.5	13.8
p	9	9	7	9
$10^3 \times \text{FI}$	2.37	1.94	2.56	1.22
	(1.00)	(1.05)	(1.64)	(0.59)
R	53.1	48.3	56.1	38.9
	(30.1)	(31.5)	(39.9)	(23.8)
$10^6 \times \epsilon_v^2$	22.6	27.7	35.9	17.3

typical of all the samples. As expected, the correlations between E_0 and the distances of the shells is quite strong. However, the correlation between distances is less than 55%, including among

Table 5. Correlation matrix for BBY S_1 model 4

Parameters are defined in Table 1 and in the text.

	E_0	r_1	r_2	r_3	r_4	α_1	α_2	α_3	α_4
E_0	1.00								
r_1 (2O)	-0.77	1.00							
r_2 (2O)	-0.77	0.41	1.00						
r_3 (1Mn)	-0.62	0.32	0.55	1.00					
r_4 (1Ca)	-0.50	0.33	0.35	0.33	1.00				
α_1	0.26	0.21	-0.65	-0.29	-0.10	1.00			
α_2	-0.04	0.62	-0.29	-0.11	0.03	0.77	1.00		
α_3	-0.08	0.18	0.05	0.02	-0.02	0.04	0.15	1.00	
α_4	-0.05	-0.05	0.08	0.11	-0.01	-0.11	-0.11	0.10	1.00

the two oxygen shells. For all of the PSII samples E_0 refines to a value between 25 and 27 eV, which is similar to that found for chemical models 1 and 2.

DISCUSSION

The recent advances in detector technology and PSII sample preparation have allowed us to improve and refine our data and our models for the manganese site in PSII. The short Mn–O distance present is characteristic of a μ -oxo bridging oxygen atom and entirely consistent with the observation of the 0.27 nm (2.7 Å) Mn–Mn distance, as previously discussed by Klein and collaborators [18,19,20,23]. We find that there are approximately two metal bridging oxygen atoms per manganese atom. Thus we conclude that, given a stoichiometry of four manganese atoms per reaction centre, the results imply the presence of two di- μ -oxo-bridged binuclear manganese units. These could be arranged either as a dimer of dimers, a tetramer or a combination of a trinuclear core and mononuclear centre. However, the latter arrangement of manganese in PSII is less likely, as the recent observation of hyperfine features on the $g = 4.1$ e.p.r. signal of NH_3 -treated PSII particles denies the possible origin of this signal as a Mn(IV) monomer and therefore removes the main reason for invoking the trimer models. The lack of any detectable Mn–Mn distance at > 0.3 nm (> 3 Å) in the present work also argues against trimer or tetramer models. Accommodation of the tetranuclear models would require significant distortions to reduce the contribution of Mn–Mn shells at > 0.3 nm (> 3 Å). Similar conclusions, based on different interpretations of the e.x.a.f.s., have been reached by Klein, Sauer and co-workers [19,20] as well as by Penner-Hahn *et al.* [28].

As both the e.x.a.f.s. and $g = 2$ and 4.1 'multilines' are minimally explained by a dimer of dimers model, such a model is favoured here.

If the scatterer responsible for the 0.37 nm (3.7 Å) shell is Ca, then this is consistent with Ca bonding to the Mn complex on the exposed luminal side. The observed Mn–Ca interaction would be too short at 0.37 nm (3.7 Å) to derive from carboxylate-bridged metal centres and is therefore probably from an oxo-bridged unit or Ca acting as a counter-ion [e.g. for concanavalin A the reported Mn(II)–Ca distance is 0.43 nm (4.3 Å) [52]]. Such units have precedence in Mn/Na mixed-metal complexes as well as in simple salts of Mn complexes with reported distances ranging from 0.30 to 0.37 nm (3.00–3.7 Å) for Na [53–55] and from 0.39 to 0.47 nm (3.9–4.7 Å) for K [56–58]; thus a 0.37 nm (3.7 Å) distance for Ca is considered possible. Ca depletion [59] and Sr replacement [59] both result in new multiline e.p.r. signals for the S_2 state, an observation expected if the Ca were intimately involved in the Mn complex. Further experiments are required to elucidate the role of Ca in water oxidation.

The small but significant changes in the structure of the Mn complex observed for the S_0^* (hydroxylamine modified S_1) $\rightarrow S_1$ [20], $S_2 \rightarrow S_3$ [19] transitions and now the $S_1 \rightarrow S_2$ transition deserve comment, the most informative changes being in the Mn–O distances. For the $S_0^* \rightarrow S_1$ transition a shortening of the bridging interaction is consistent with the change in metal oxidation state and, perhaps, deprotonation of a bridging ligand. However, on increasing the Mn oxidation state on the $S_1 \rightarrow S_2$ transition the Mn–O distances apparently become longer [0.003 and 0.006 nm (0.03 and 0.06 Å)]. For the $S_2 \rightarrow S_3$ transition there is an apparent increase in the bridging distance [19], apparently with constant Mn oxidation. Clearly deprotonation of ligands and increasing metal oxidation state should result in a decrease in the average ligand distances unless some major structural rearrangement occurs involving the oxygen ligands. Thus the $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ transitions probably do not just involve storage of oxidative equivalents.

Conclusions

We have been able to confirm the presence of the short low-Z scatterer at ≈ 0.18 nm (≈ 1.8 Å) and report the existence of a shell of scatterers at 0.37 nm (3.7 Å).

An apparent lengthening of the Mn–O distances occurs on going from $S_1 \rightarrow S_2$ states contrary to expectations based on simple oxidation of Mn.

Analysis of the e.x.a.f.s. indicates a Mn–Ca interaction is primarily responsible for the 0.37 nm (3.7 Å) shell, although it is not possible to exclude totally other single- or multiple-shell fits at this distance.

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