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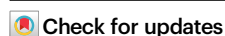
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An additional water is introduced into the manganese cluster during the formation of the S₃ state of photosystem II

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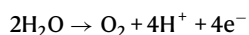
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During photosynthetic water oxidation, the Mn₄Ca cluster in Photosystem II progresses through five intermediate S_i (i = 0–4) states. X-ray crystallography studies have reported the insertion of one new O ligand during the formation of the S₃ state, but recent studies question the presence of this additional ligand based on cryo-EM and earlier room-temperature crystallography data. There is also controversy about whether the O–O bond interaction already occurs in the S₃ state or in the subsequent S₃ to S₀ transition. Here we report conventional high-resolution data for the S₁, S₂, and S₃ states to a resolution of ~1.9 Å, and anomalous diffraction data at two energies (9.5 keV and 7 keV), that was used to model the Mn positions, followed by determination of oxygen positions using the high-resolution maps. We show that the new oxygen atom, O_x (or O6), in the S₃ state is observable as a distinct peak without any restraints, confirming its ligation to Mn1 and Ca. The O_x–O5 distance is ~2.1 Å, supporting no strong interaction between them in the S₃ state, suggesting that if this is the O–O bond formation site, it is formed during the S₃ to S₀ transition initiated by the final oxidation of the cluster.

In oxygenic photosynthesis, energy from sunlight is harnessed and converted to chemical energy in green plants, algae and cyanobacteria, through a chain of reactions¹. The very first step of these reactions takes place in Photosystem II (PS II), a multi-subunit membrane protein complex, where electrons and protons are extracted from water using light, as follows:



PS II carries out this reaction by coupling the four-electron oxidation of water at the oxygen-evolving complex (OEC—the Mn₄Ca cluster and surrounding water and amino acid residues), with the one-electron photochemistry occurring at the reaction center, where light is absorbed. It cycles through five intermediate S-states (S₀ to S₄) that correspond to the abstraction of four successive electrons from the OEC (Fig. 1)^{2,3}. During this multi-photon induced, multi-electron catalysis, spatially and temporally controlled transport of electrons,

protons, and water substrate to and from the OEC is essential for maintaining the intactness, efficiency, and turnover of the catalyst⁴.

The recent advent of characterization tools such as serial femtosecond crystallography and cryo-electron microscopy (cryo-EM) have been very consequential. Following extensive efforts in traditional synchrotron X-ray crystallography at cryogenic temperatures (for example^{5–7}), X-ray free-electron laser (XFEL)-based crystallography at room temperature has had a profound influence on the structural and mechanistic studies of the water oxidation reaction. The method has enabled researchers to follow the structural and chemical changes under the enzyme's functional conditions^{8–13}, leading to the crystal structures of the intermediate states (S₀, S₁, S₂, and S₃) at ~2 Å, and also to the observation of structural changes during S-state advancements^{10,14–16}. Furthermore, single particle cryo-electron microscopy has made it possible to investigate structures of PS II from diverse organisms^{17–22}. However, conclusions about the precise structure of the Mn₄Ca cluster in its various S-states are presently not possible from cryo-EM studies due to uncertainties

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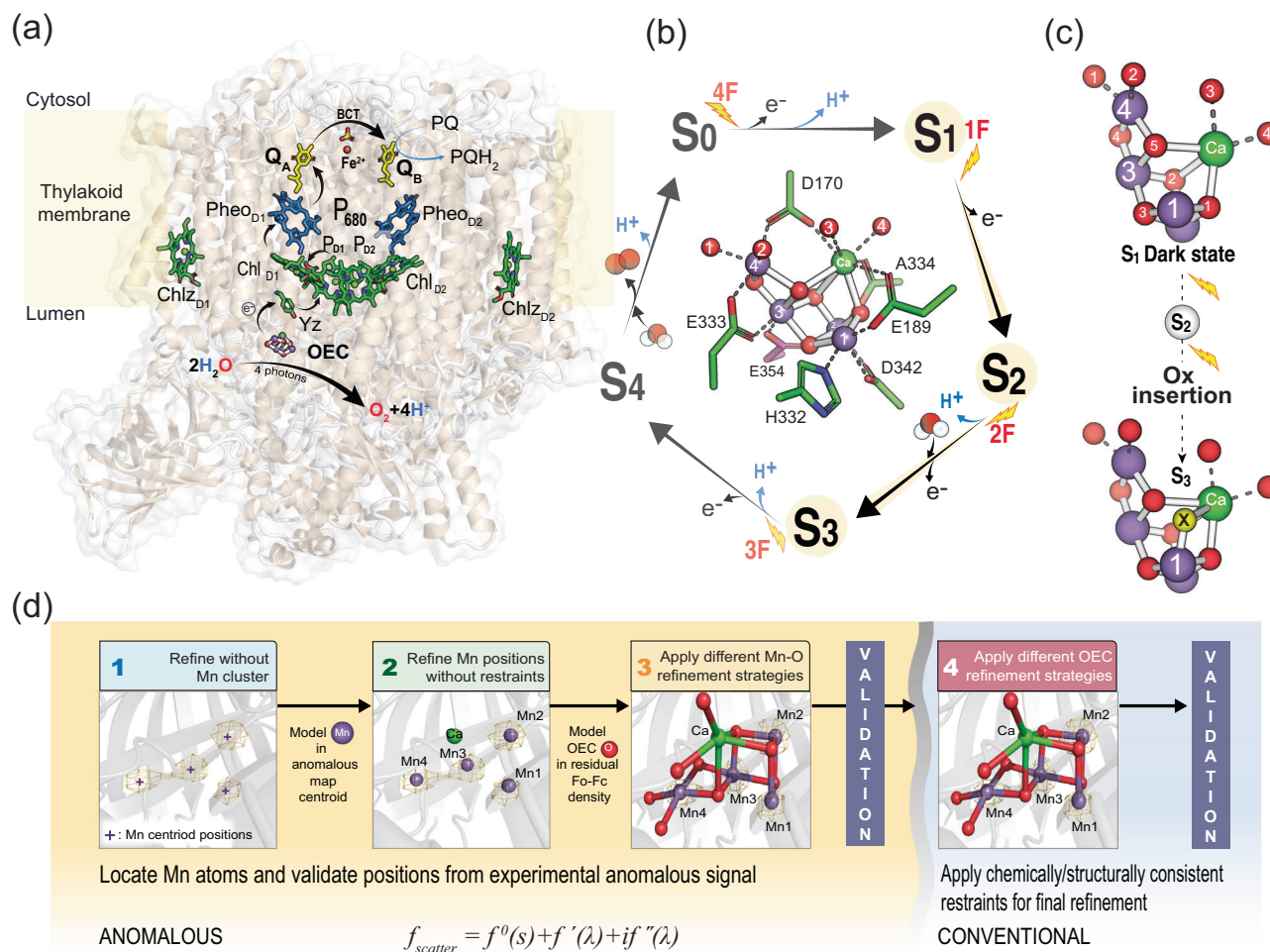


Fig. 1 | Overview of photosystem II (PS II), the oxygen evolving complex (OEC) and the Kok cycle and the refinement workflow used in this paper. a Overall structure of PS II embedded in the thylakoid membrane. The electron transfer chain is highlighted in color. It consists of the initial charge separation region (P₆₈₀ reaction center chlorophylls), the electron acceptor side (Pheophytins–Pheo, Quinones–Q_A, Q_B and associated components) and the electron donor side that includes redox-active Y_Z and the OEC where the water oxidation reaction takes place. **b** The OEC is a Mn₄Ca cluster with several bridging oxygens and is ligated to several waters and amino acids in the luminal side of PS II. The Kok cycle is the

4-step water oxidation cycle taking place at the OEC driven by the sequential 1 photon absorption (1F through 4F) in the P₆₈₀ region of PS II. **c** Structure of the OEC in the S₁ and S₃ states from previous work^{10,14} show binding of an additional water molecule (O_x or O₆) upon application of 2 laser flashes. Mn centers are shown in purple, O_x in yellow, O in red, and Ca in green. **d** Structural refinement workflow to obtain the final OEC structures shown in this paper. Both anomalous and conventional diffraction data were used to model the Mn locations and the O positions precisely. We also validated the restraints used in the refinement. See Supplementary Fig. 7 for a more detailed figure.

regarding the S-state population and the well-documented radiation damage to the Mn₄Ca cluster during data collection^{20,22}.

A key structural insight gained from XFEL crystallography in PS II is the visualization of a new oxygen ligand at the OEC during the S₂ to S₃ transition^{10,12,13}, followed by its disappearance when transitioning from the S₃ to the S₀ state^{10,15}. The results show that a newly inserted ligand (O_x or O₆) in the catalytic center can be distinctly identified in the room temperature crystal structures taken at various time points during the oxidative process, and its later disappearance in the S₃ to S₀ transition indicate the involvement of this ligand in the O–O bond formation and the release of molecular oxygen. While XFEL structural studies point to the presence of this new ligand in the S₃ state^{10,12–15}, there is ongoing debate about whether this ligand is even present and if present what is the chemical nature of this oxygen, *i.e.*, whether it exists as hydroxo (HO[–]), or oxyl (O[•]) in which this oxygen interacts with the neighboring O5^{13,23–25} forming a putative O–O bond. We also note that there is ongoing controversy regarding the interpretation of the XFEL serial crystallography data and whether it is sufficiently robust to confirm the presence of this additional oxygen in the S₃ state^{26,27}. Recent theoretical studies challenge the XFEL crystallography data

analysis on the basis of a perceived mismatch of assigned Mn oxidation states and reported Mn–Mn, Mn–O, and Mn–ligand distances^{28,29}. While the structural refinement process in crystallography involves multiple intricate steps, typically only the final structural models are presented. This makes step-by-step validation of the refinement process and reproducibility and reliability of the structural models challenging. In particular, modeling metal cofactors in metalloenzymes, which often requires chemically meaningful stereochemical restraints, could dictate atomic distances in the final model and potentially limit the flexibility required to accurately represent biological systems.

The main goal of this study is to resolve the ongoing debate over the structure of the S₃ state, a key intermediate formed after the second laser flash from the dark S₁ state using two sets of data. Anomalous diffraction data first provided a validation for the location of the Mn atoms (Fig. 1d). For the second step, we then used higher-resolution conventional diffraction data (~1.9 Å), which reduces the modeling error and the reliance on restraints compared to previous studies at lower resolutions (2.1 Å). Combining the results precisely define the positions of the bridging O atoms and conclusively shows the presence of an additional ligand in the S₃ state. We further discuss the chemical

nature of the O_X ligand and its role in the O-O bond formation mechanism. We show that the O_X -O5/other O-atom distances are incompatible with the O-O bond formation in the S_3 state, thereby supporting the formation of such bond during the S_3 to S_0 transition.

The method we describe here for evaluating the effect of chemical restraints and estimation of coordinate errors in the derived OEC model is also widely applicable to other metalloenzymes. An important goal of many such studies^{30,31} is to detect and evaluate subtle yet functionally important structural changes in time-resolved crystallography data in which a multi-metal catalytic center goes through complex redox changes. The techniques presented here address several common challenges in interpreting functional data such as disentangling multiple components and modeling light atoms coordinated to metal centers.

Results

Use of anomalous signals for determining accurate Mn positions in the OEC

XFEL crystallography data for the dark stable state (OF, predominantly in the S_1 state), as well as the one flash (1F, 90% S_2) and two flash (2F, 65% S_3 and 35% S_2 , see Methods for population estimates) data for PS II were collected at an X-ray energy of 9.5 keV and merged to a resolution of 1.97 Å, 1.98 Å and 1.93 Å, respectively. Details of the data collection are described in the Methods section. We first assess and validate the accuracy of the Mn positions in each S-state using anomalous electron density maps (see below for explanation), including additional anomalous data collected at an X-ray energy of 7 keV. This approach allows deconvolution of the contribution by Mn to the electron density and thereby making it easier to identify the O positions. Subsequently, we present multiple lines of evidence for O_X insertion in the 2F state using both anomalous and conventional diffraction data. Lastly, the modeling of oxygen positions in the Mn_4Ca cluster is analyzed along with changes in the Mn/Ca ligand environment. We note that PS II is crystallized as a homo-dimer and out of the two PS II monomer sites (monomer I and II, see Supplementary Notes 2 for definition), the results from monomer I are discussed here. While there are small structural differences between the two monomers due to the packing environment^{7,32}, the overall structural changes in monomer II are very similar to those of monomer I, and their equivalent of Figs. 2, 3 and 4 are provided in the Supplementary Figs. 2–4.

Figure 2a compares the regular $2F_O - F_C$ map with the anomalous maps around the Mn sites. The $2F_O - F_C$ map was generated using conventional intensities (i.e., $I_{obs}(+)$, $I_{obs}(-)$, were averaged), which represents the conventional method in X-ray crystallographic analysis for placing atoms such as Mn and O. In contrast, the anomalous maps were generated by the difference in the intensity of the Friedel pairs, ($I_{obs}(+)$, $I_{obs}(-)$). Metal atoms such as Mn in PS II scatter X-ray strongly and the signal intensity in the anomalous diffraction map depends on the atom's anomalous scattering coefficients (f'' , see Fig. 1d). Other key factors include resolution, flexibility, as well as experimental errors. The intensity of f'' is a function of X-ray energy; at 9.5 keV, f'' of Mn is ~ 2.0 e⁻ which is about 50% of its maximum intensity at the Mn K-edge (6.56 keV) (Fig. 2b) whereas the intensity of f'' for carbon or oxygen is only ~ 0.01 e⁻. Therefore, metals such as Mn dominate the signal in anomalous maps at the X-ray energies studied here and the anomalous diffraction signal ($\Delta_{ano} = |F_{obs}(+)| - |F_{obs}(-)|$), where F is the complex form of the structure factor, $I_{obs} \sim |F_{obs}|^2$) is the most sensitive measure for identifying the location of metals. The power of anomalous diffraction data is demonstrated by their use in de novo phasing methods in X-ray crystallography, enabling the precise localization of metal ions within the unit cell without requiring prior knowledge of the protein structure. In the approach presented here, we are able to identify the Mn positions without any prior information about their placement in the structure, minimizing model bias.

The anomalous map peak heights at all 4 Mn atoms exceed 9σ , with Mn1 and Mn2 showing the highest peak heights (11–12 σ) (Supplementary Figs. 5), and Mn4 showing the lowest peak height with the highest B-factors. The comparison of the $2F_O - F_C$ map with the anomalous maps agrees well within the coordinate error of refinement³³ as well as the grid spacing used for both maps (Fig. 2a top). Furthermore, the Mn-Mn distances calculated from the centroids of Mn density in both maps match closely (Supplementary Table 5). The results confirm that the Mn positions determined from conventional map analysis and refinement are consistent with those obtained from anomalous data. The pure S-state refined models (after deconvolution of the flash data based on population analysis; see modeling approach in Methods) are overlaid in Fig. 2a. The Mn-Mn distances obtained from the current data set at 9.5 keV are shown in the 2nd row of the figure (see also Supplementary Fig. 1 for alternate view of anomalous density). In the S_1 to S_2 transition, we observe a slight shortening of the Mn3-Mn4 distance from 2.78 ± 0.03 Å to 2.72 ± 0.03 Å. The errors reported in this paper, unless otherwise stated, were calculated using the END/RAPID procedure (see Supplementary Methods). In addition, changes to the ligation environment of Mn4 are also observed (see section on 'Mn-O distances in the cluster' below). These observations are compatible with the oxidation of the Mn4 site. In the S_2 to S_3 transition, an expansion of the OEC cluster is observed, with the increased distances of the Mn1-Mn3 (3.25 ± 0.04 Å to 3.41 ± 0.03 Å) and Mn1-Mn4 (4.86 ± 0.03 Å to 5.14 ± 0.03 Å) pairs. The Mn-Mn distances from anomalous density for the three di- μ -oxo bridges in the S-states are comparable to those from extended X-ray absorption fine-structure (EXAFS) data³⁴ (2.7–2.8 Å, see Supplementary Table 10). In the S_1 to S_2 transition, the EXAFS measurement shows a shortening of one of the di- μ -oxo bridges, consistent with contraction of the Mn3-Mn4 bridge in the crystallography data. While the ~ 3.3 Å bridge is observed in the S_1 and S_2 states from both techniques, a significant departure happens in the S_3 state where the XFEL data shows an expansion of the Mn1-Mn3 bridge (~ 3.4 Å) that is not observed in the EXAFS model. This can be attributed to the contribution from the Mn-Ca bridges which are in the same distance range, making it hard to deconvolute in the EXAFS data.

The elongation of the Mn1-Mn4 distance can be visualized in the map by plotting a 1D trace along the Mn1-Mn4 vector, starting from the Mn1 position in each S-state (Fig. 2c). The expansion of the Mn_4Ca cluster in the 2F state can be independently confirmed by calculating isomorphous difference maps using the conventional diffraction data. These $F_O - F_O$ maps are calculated between states A and B, denoted as B-A maps in this paper, by taking the difference in measured intensities for the 2 states and a common phasing model (from state A). Thus, they have minimal model bias and can identify structural differences between the states without detailed refinement. The data for the 2F - 1F map shown in Fig. 3a (right side) shows the expansion of the OEC cluster (features C, D, F; see Supplementary Note 1), in line with previous publications^{10,13–15}. The data for the 1F - OF map (Fig. 3a, left side) does not show any evidence for an expansion of the cluster.

The above observation was further confirmed by collecting anomalous diffraction datasets for the OF and 2F states at 7.0 keV, closer to the Mn K-edge energy (Fig. 2b). At 7.0 keV, the f'' of Mn is 3.5 e⁻ which is about 85% of the maximum anomalous dispersion coefficient value. Consequently, the Mn anomalous signal in the electron density maps is higher compared to that at 9.5 keV. The modeling procedure was similar to the 9.5 keV case and further details are provided in the "Methods". The 7 keV results independently corroborate the 9.5 keV observations—we see an elongation of the Mn1-Mn4 (4.82 ± 0.03 Å $\rightarrow$ 5.06 ± 0.04 Å) and Mn1-Mn3 (3.19 ± 0.04 Å $\rightarrow$ 3.36 ± 0.04 Å) distances in the 2F state. This is evident both in the anomalous map centroid positions (Supplementary Table 5) as well as in the final S_3 state model (Fig. 2b, c trace). The elongation of the Mn1-Mn4 and Mn1-Mn3 distances is consistent with the insertion of the new ligand (O_X) to bridge Mn1 and Ca, which we discuss in the following section.

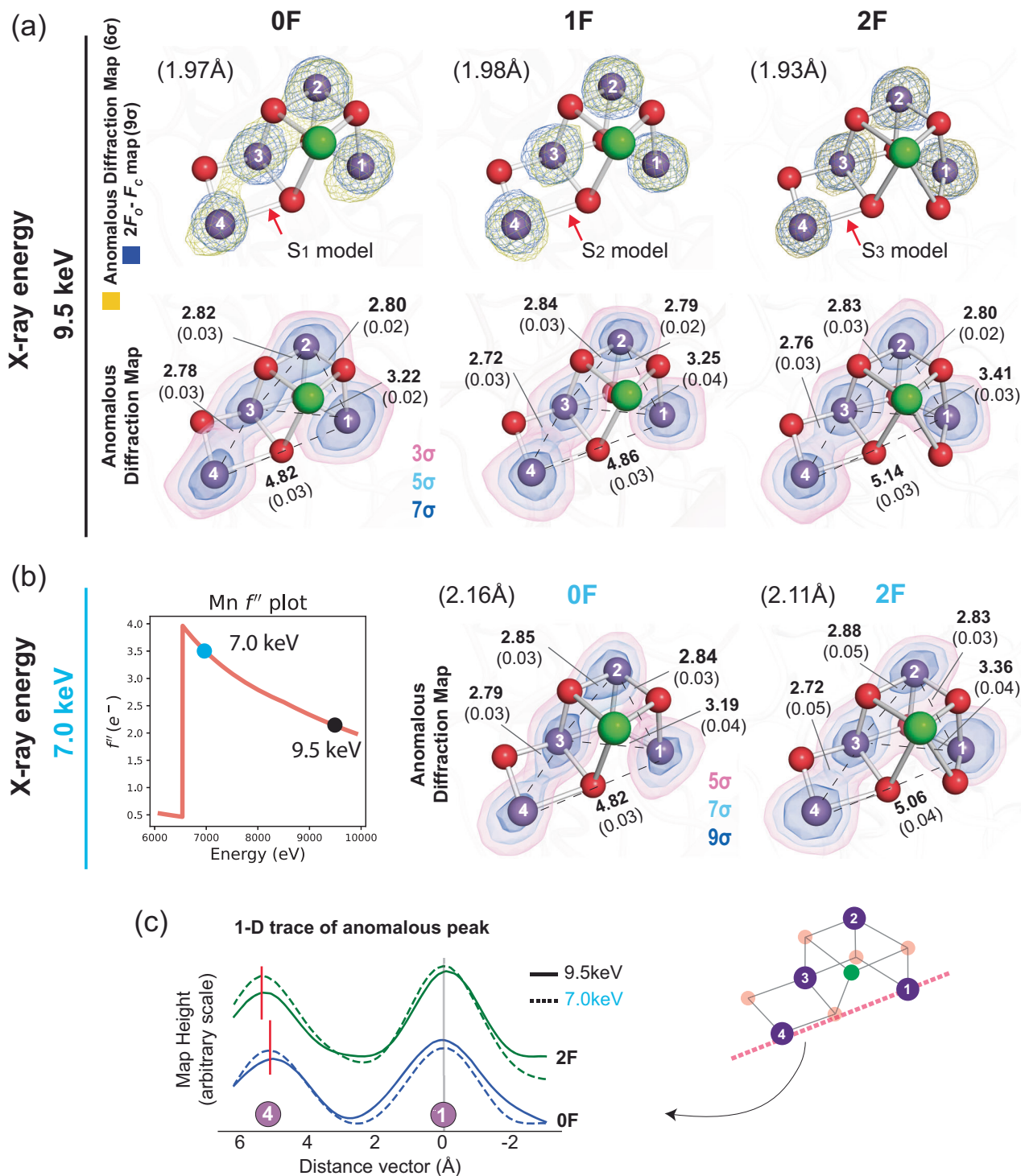


Fig. 2 | Accurate Mn positions in PS II with anomalous diffraction data and evidence for Mn_4Ca cluster expansion in S_3 state. **a** Upper panel: Overlay of anomalous diffraction maps at 9.5 keV and standard $2F_o - F_c$ maps highlighting the degree of similarity of the two maps in all 3 S-states. Lower panel: Anomalous diffraction maps at 9.5 keV for the Mn_4Ca cluster in the 0F (S_1), 1F (S_2) and 2F (S_3 majority) states. Mn-Mn distances shown are for the model refined with anomalous intensities and error estimates are from the END/RAPID procedure. The Mn-Mn distances were subsequently restrained tightly to the values from the anomalous

data when we modeled oxygen positions with conventional intensity data. **b** Anomalous diffraction maps at 7.0 keV for the Mn_4Ca cluster in the 0F (S_1) and 2F (S_3 majority) states. Distances shown are for the model refined with anomalous intensities. At 7.0 keV, the f'' value of Mn is approximately 3.5 e⁻. **c** 1-d trace of the anomalous diffraction map along the Mn1-Mn4 vector with the origin being the modeled Mn1 position. A shift in the relative Mn4 position can be seen, confirming expansion of the Mn_4Ca cluster in the S_3 state. Refer to Supplementary Fig. 2 for results from monomer II. Source data for panel c is provided in the Source Data file.

Evidence for the presence of O_x in the S_3 state

Following the confirmation of cluster expansion in the S_3 state using anomalous data, we provide evidence for a new ligand at Mn1, based on a clear electron density signal from the higher resolution

conventional diffraction data. We first consider isomorphous difference maps between the consecutive flash state datasets (Fig. 3a). Details of the difference features in the maps are discussed in Supplementary Note 1. In the 2F state, we observed electron density

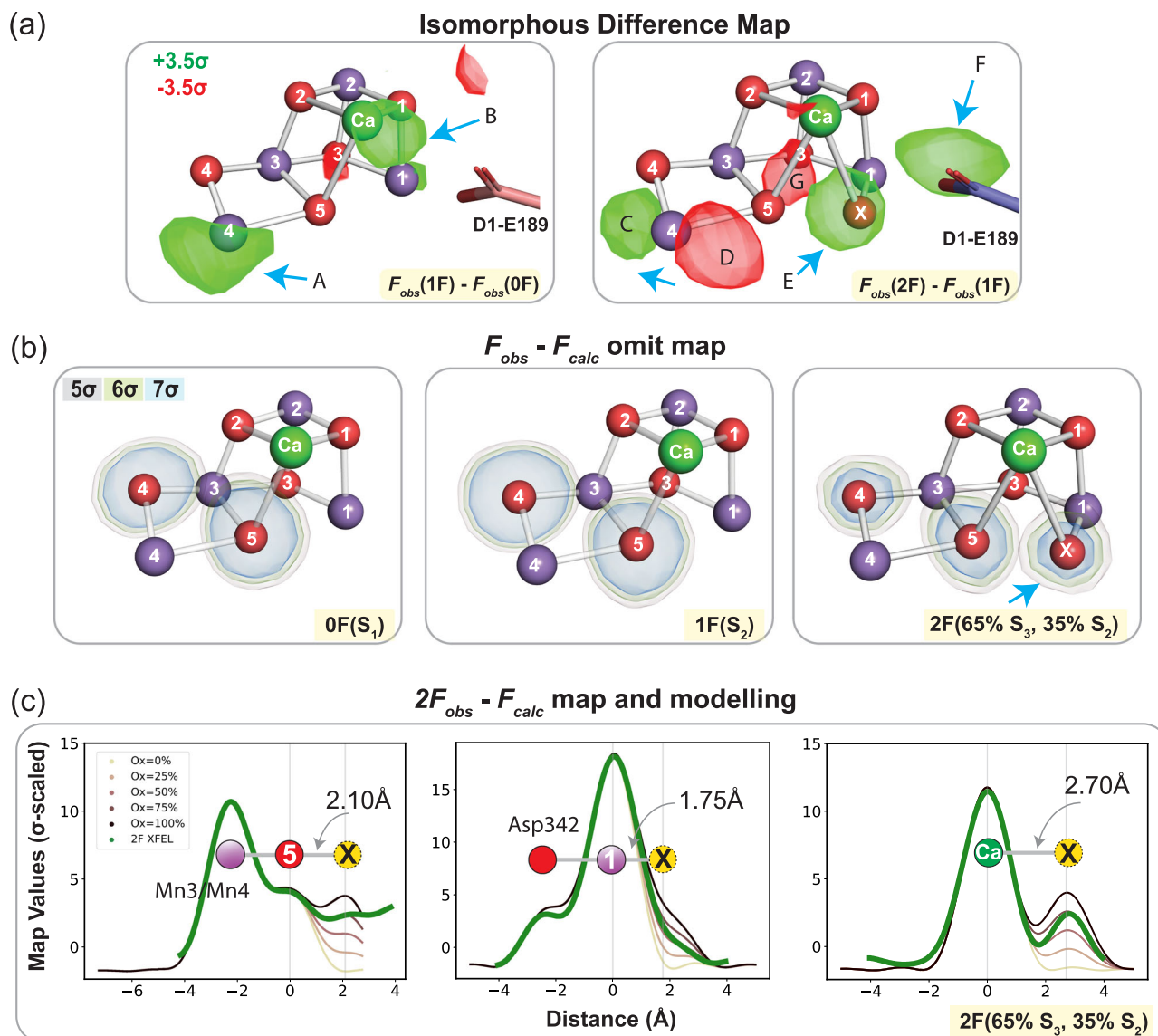


Fig. 3 | Evidence for insertion of O_X in the S_3 state based on combining anomalous and conventional data. **a** Isomorphous difference maps ($F_O - F_C$) for the 1F - 0F and the 2F - 1F datasets contoured at $\pm 3.5\sigma$. The O_X signal shows up as a 6.5σ peak in the 2F - 1F difference map. Overlaid are the S_2 and S_3 structures in the respective figures. For discussion see text and Supplementary Note 1. **b** $F_O - F_C$ omit maps for the O4, O5, and O_X atoms in the S -states with the O_X omit peak height being 8.5σ . Overlaid are the S_1 , S_2 , and S_3 structures. **c** 1-D traces of the $2F_O - F_C$ experimental map for the 2F state along the bond vectors (O5- O_X , Mn1- O_X , Ca- O_X) and comparison with theoretical X-ray scattering map values from 2- or 3-atom

models. In the simplified models, the O_X population was varied between 0 and 100%. An O_X population between 50 and 75% in the theoretical curves best match the 2F state experimental profiles, supporting its modeling. A more precise estimate of 65% O_X occupancy used in the modeling of the 2F state is obtained from omit maps and multiple spectroscopic techniques. Additional details of the plots in panel c are provided in the Supplementary Methods. Refer to Supplementary Fig. 3 for results from monomer II. Source data for panel (c) is provided in the Source Data file.

corresponding to the additional ligand (O_X), located near Mn1 and Ca ions (feature E). This density is detected in the isomorphous 2F - 1F difference map as a 6.5σ peak. No such peak is observed in the preceding S_1 - S_2 transition. We also observe movement of the Mn4 (positive/negative pair of density, features C, D) as well as evidence for slight movement of the Mn1 and Glu189 residue (features F, G). The movement of the Mn centers in the 2F - 1F difference map suggests an expansion of the Mn_4Ca cluster in the S_3 state, which is consistent with the observations from the anomalous data in Fig. 2. To validate the presence of O_X in the 2F data, we calculated $F_O - F_C$ omit maps for 0F, 1F, and 2F data (Fig. 3b) and compared the O_X intensity in these maps with that of other bridging oxygens (O4 and O5) in the Mn_4Ca cluster (see Supplementary Fig. 8). Omit maps are a common tool to verify if a region of interest in the structure is supported by the experimental

data. By excluding the atom(s) of interest, a residual $F_O - F_C$ map is calculated. If the model is supported by the data, the electron density in the region of the omitted atoms is expected to show a positive signal. The data here supports the presence of the new ligand bridged between Mn1/Ca ions in the 2F data with a $F_O - F_C$ omit peak height of 8.5σ , in agreement with previously published work^{10,12-15}.

More direct evidence for the presence of O_X is seen in the $2F_O - F_C$ electron density maps (Fig. 3c). The improved resolution of the current 2F data close to $1.9 \text{ \AA}$ enabled us to detect signal for O_X unambiguously in the $2F_O - F_C$ electron density maps. In crystallography, $2F_O - F_C$ maps are considered to be the best estimate of the true electron density from diffraction data with the least model bias and lower errors. Therefore, a significant electron density peak exceeding $1-1.5\sigma$ (where σ represents the RMS variation of the electron density) detected in the

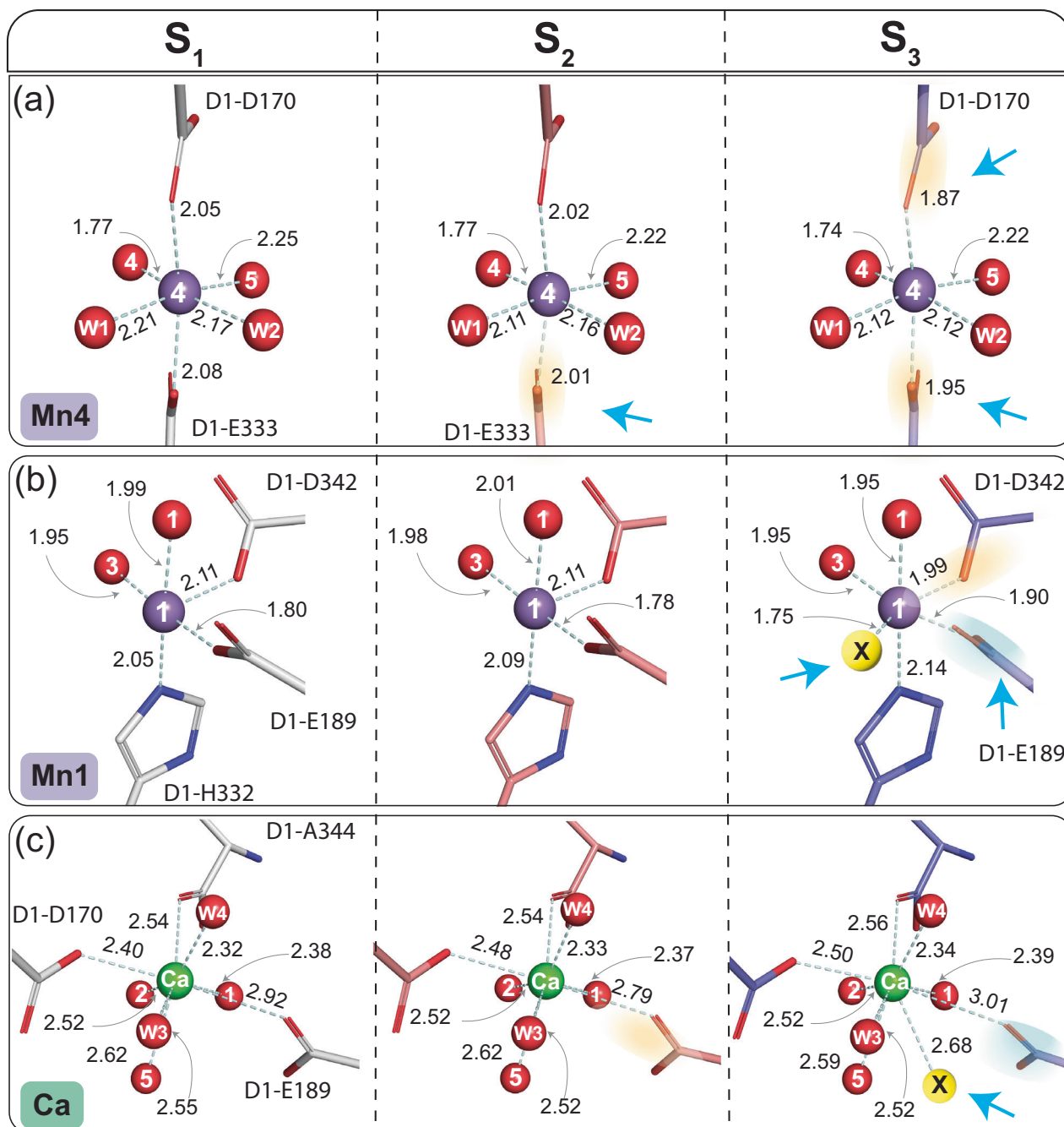


Fig. 4 | Mn and Ca ligand environment in the S_1 , S_2 , S_3 states and coordination bond distances. The OEC cluster and active site residues form coordination bonds with the Mn/Ca atoms. Shown are the coordination environment for (a) Mn4 (b) Mn1, and (c) Ca sites where the major structural changes occur. Mn, Ca, and O atoms are shown as purple, green, and red spheres, respectively. The new inserted

water in the S_3 state, O_X , is shown in yellow. Blue arrows denote the main changes observed. Significant distance contractions are highlighted with yellow shade and distance expansions with blue shade. The ligand environments of Mn2 and Mn3 are shown in Supplementary Fig. 6. Refer to Supplementary Fig. 4 for results from monomer II.

maps would provide strong evidence for the presence of an additional atom, analogous to the assignment of water molecules elsewhere in the structure. In the $2F_o - F_c$ map, the extra peak close to Mn1 has a height of 2.6σ , indicating the presence of the new ligand O_X . In Fig. 3c, we show 1D map profiles between O_X and neighboring atoms (O5, Mn1, Ca) for the 2F data. These 1D profiles were then compared with calculated map profiles from linear two or three-atom models (Mn(3,4)-O5- O_X or O_{D342} -Mn1- O_X or Ca- O_X) where the distance/B-factor parameters for the oxygen atoms were kept fixed close to the S_3 model (see Supplementary Methods for exact values used). While these simplified models do not account for the contribution from every atom in the

OEC, their simplicity allows us to evaluate the effects of the presence/absence of O_X . In these simulations, the occupancy of O_X in the S_3 model was varied from 0 to 100% and compared with the experimental data. We observe a peak for O_X in both the Ca- O_X and O5- O_X experimental trace as well as a shoulder in the Mn1- O_X trace. Such a peak in the $2F_o - F_c$ profile supports the presence of an additional oxygen, as seen in the calculated map traces. Comparison of the profiles suggests an occupancy of O_X between 50 and 75% which is in the range of our estimate of 65% for the population of the S_3 state in doubly flashed samples from spectroscopic data¹⁰ and matches well with expected yields of the S_3 state based on excitation efficiency in PS II samples^{35,36}.

The B-factor of O_X ($\sim 30.5 \text{ \AA}^2$) is comparable to other oxygen ligands (including $O5$ - $28.3 \text{ \AA}^2$) in the OEC and surrounding ordered waters (Supplementary Table 9), indicating that its occupancy and placement are consistent with the local environment. The $O5$ peak height is virtually unchanged in the 2F data compared to the 0F/1F data, ruling out any displacement of $O5$ to a position in-between $Mn1$ and $Mn4$ (see Supplementary Fig. 8 for omit map peak heights). Separate refinement of the 2F model with the Flat-bottom Harmonic restraints (FBHR, see step 4b in Supplementary Table 4 and Supplementary Fig. 10) resulted in negligible change in the $O5$ position, even in the absence of O_X , confirming that the $F_O - F_C$ difference peak cannot be explained by a shift of $O5$. We also checked how sensitive the O_X signal is to phases from the surrounding active site. This was done by calculating Polder omit maps from phases obtained by deleting all first shell residues, waters, and the OEC, randomly perturbing the remaining model and subsequent long refinement (100 macrocycles) in order to remove memory of the initial phases. The results, presented in Supplementary Fig. 17, show features for an additional ligand in the 2F map. All the above evidence thus supports the insertion of a new water, O_X , in the S_3 state.

Mn-O distances in the cluster

Besides confirming the presence of O_X (or $O6$) in the S_3 structure, our workflow using the improved resolution of crystallographic data helps model oxygen positions more precisely in the OEC. In this step, we used conventional intensities for further structural refinement as they have a higher signal-to-noise ratio compared to the anomalous intensities (see Supplementary Table 1) due to the averaging of the anomalous pairs and are more suitable to model lighter atoms in the vicinity of metal sites. The Mn positions were tightly restrained based on the anomalous refinement (see Methods for details). Due to this, the oxygen atom positions could be refined with minimal influence from the adjacent Mn sites. Figure 4 shows the ligand environment for the two important Mn centers ($Mn1$ and $Mn4$). In order to derive the optimal model for the Mn-O distances, we thoroughly examined the effect of restraints which we discuss first.

In crystallographic refinement, restraints are necessary to refine the atomic model of a structure when the data does not extend to atomic resolution (*i.e.*, better than $1.0 \text{ \AA}$). Therefore, almost all protein structures are refined with geometric restraints that help to maintain their chemical integrity. While the Mn positions are well determined from standard $2F_O - F_C$ and anomalous diffraction maps (as shown in Fig. 2a), the position of oxygen atoms in the vicinity of Mn still needs to be restrained for the stability of refinement at our current resolution. For example, as shown in Supplementary Fig. 10, using extremely loose restraints can often lead to the refinement falling into a local minimum with large difference map features and unphysical geometry. Thus, while the experimental data alone provides evidence for placement of the oxygen atoms in the structure, restraints are needed to ensure they stay close to the density peak during the refinement process. Our group's previous work has used harmonic restraints based on prior information from X-ray absorption spectroscopy of PS II and model complexes as well as small molecule crystallography data (used in refs. 10,14). We had used custom restraints for the OEC with $0.05 \text{ \AA} \leq \sigma \leq 0.10 \text{ \AA}$ for the bond lengths¹⁰, which is looser than most standard restraints for metal clusters found in crystallographic software packages, including for the Mn_4Ca cluster in PS II ($\sigma = 0.02 \text{ \AA}$ as found for OEX/OEY in the CCP4 monomer library)³⁷. The restraints we previously used provided sufficient weight to the experimental data for determining the oxygen position while still maintaining a physically plausible geometry for the cluster. A comparison of the different restraint sets is shown in Supplementary Fig. 13. In this work, enabled by improved resolution and use of anomalous data, we developed a set of restraints (Supplementary Fig. 7).

We used the anomalous data to obtain the Mn positions and Mn-Mn distances (Step 1, 2, 3a in Supplementary Fig. 7) and used harmonic restraints (HR) to define the bridging Mn-O distances ($\sigma = 0.05 \text{ \AA}$, Step 4a in Supplementary Fig. 7) in the cluster. We note that the Mn-amino acid links are defined using extremely loose restraints ($\sigma = 0.50 \text{ \AA}$, see Methods), providing virtually no penalty. No information regarding oxidation states is used during the refinement process. Multiple validation checks were done to confirm that the restraints do not bias the OEC model. This included quantifying the restraints penalty during refinement, $F_O - F_C$ difference map flatness and running independent refinements with flat-bottom harmonic restraints (FBHR, Step 3b and 4b in Supplementary Fig. 7; also see "Methods"). In FBHR, the penalty over a wide range of Mn-O distances (1.6 – $2.3 \text{ \AA}$) is zero (flat bottom part) with a harmonic ramp up beyond this domain ($\sigma = 0.05 \text{ \AA}$). Full details of the validation procedure are provided in Supplementary Methods. The results show no evidence of the HR restraints distorting the OEC geometry towards a conformation inconsistent with the X-ray data. For example, the final RMS deviation of the OEC cluster distances between HR and FBHR restraints (step 4a vs step 4b) is $-0.05 \text{ \AA}$ (Supplementary Table 4). As a control, the same checks were also done for the default restraints in the CCP4 monomer library ($\sigma = 0.02 \text{ \AA}$). In this case, the OEC model was highly strained with strong disagreement with the X-ray data, suggesting the default restraints are not suitable. By contrast, calculations with the previously used restraints from our group^{10,14} showed excellent agreement with the HR restraints developed in this paper with an RMS deviation between the two methods of $\sim 0.02 \text{ \AA}$ (Supplementary Table 4). All results shown in this paper were obtained with our current set of harmonic restraints (Step 4a in Supplementary Fig. 7).

In the refined final models, the $Mn-O_{\text{bridge}}$ distances are less than $2.0 \text{ \AA}$ and $Mn-W1/W2$ distances are longer (2.1 – $2.2 \text{ \AA}$, except for the $Mn4-O5$ distance which we discuss later), while the $Mn-O_{\text{carboxyl}}$ distances range between 1.8 and $2.1 \text{ \AA}$. The Ca interactions with oxygens are longer in the range of 2.3 – $2.9 \text{ \AA}$. Overall, these bond lengths are consistent with those observed in inorganic complexes.

Figure 4 shows the ligand environment of two Mn centers, $Mn1$ and $Mn4$. These two Mn are considered to go through the oxidation state changes during the S_1 to S_3 transitions (further details in Supplementary Fig. 6). The major change in the S_1 to S_2 transition was seen on the $Mn4$ site where a shortening of the $Mn4 - O_{D1:E333}$ distance is observed (from $2.08 \pm 0.05 \text{ \AA}$ to $2.01 \pm 0.04 \text{ \AA}$). This observation is consistent with our previous 1F XFEL structures (PDB: 6DHF). Interestingly, the $Ca - O_{D1:E189}$ distance also shortens in this transition ($2.92 \pm 0.06 \text{ \AA}$ to $2.79 \pm 0.04 \text{ \AA}$).

In the subsequent S_2 to S_3 transition, changes at the $Mn1$, $Mn4$ and Ca sites are observed, associated with insertion of the new ligand, O_X . On the $Mn1$ side, a contraction of the $Mn1-O_{D1:D342}$ distance ($2.11 \pm 0.04 \text{ \AA}$ to $1.99 \pm 0.06 \text{ \AA}$) is seen on the opposite side of the $Mn1-O_X$ axis. The E189 residue moves away from the cluster to accommodate the insertion of the new ligand (positive density in Figs. 3a and 4), resulting in elongation of the $Ca1-O_{D1:E189}$ distance ($2.79 \pm 0.04 \text{ \AA}$ to $3.01 \pm 0.08 \text{ \AA}$). With the insertion of O_X in the S_3 state, the $Mn1$ is in a 6-coordinate octahedral environment. On the $Mn4$ side, a shortening of the $Mn4-O_{D1:D170}$ distance is seen ($2.0 \pm 0.04 \text{ \AA}$ to $1.87 \pm 0.05 \text{ \AA}$), suggesting adjustments during the movement of $Mn4$ when the cluster expands.

In all 3 S-states, a long $Mn4-O5$ distance ($2.2 \text{ \AA}$) is observed, regardless of the restraints parameters used (Supplementary Table 3 and 4). No evidence for a shorter distance ($< 1.9 \text{ \AA}$), as predicted by the DFT calculations²⁸, is found in the electron density map. Thus, our experimental data supports assignment of a longer $Mn4-O5$ distance, consistent with our previous XFEL structures^{10,14,15}.

Comparison with previous S_1 and S_2 state structures

The high-resolution of the S-state structures allowed us to evaluate S_1 to S_2 changes in the OEC and the ligand environment. The overall

agreement of distances in the ligand environment with previous structures^{10,14} is excellent. The improved resolution and modeling here lead to lower error in the Mn–O bond distances (Supplementary Table 6). In the S_1 state, the Mn1 is in a pentacoordinate geometry while the other three Mn sites are hexacoordinate. The OEC geometry remains mostly unchanged in the $S_1 \rightarrow S_2$ transition with changes in the Mn4 ligand environment as described above. The Mn1 remains pentacoordinate in the S_2 state. This open cubane-like geometry in the S_2 state agrees well with previous structures and is consistent with models of low spin S_2 ($S_{\text{total}} = 1/2$) with a bridge between Mn4 and O5, with no Mn1–O5 bond. Also, there is no indication for a closed cubane-like geometry with a Mn1–O5 bridge in the current model, in agreement with previous data. Refinement done with different restraints (Supplementary Table 4), show no propensity for a secondary O5 conformation closer to Mn1 and this was further supported by the O5 omit map feature (Fig. 3b).

Changes in the water and hydrogen-bond network

In the second coordination sphere of the OEC, we observed several notable changes in the water and hydrogen-bond networks (Supplementary Fig. 11). In the S_1 – S_2 transition, a strong negative isomorphous difference density 1F–OF is observed at the W20 position in the O4 channel (feature B), indicating its disappearance or increased disorder, consistent with previous observations^{9,10,13,14,38,39}. As a result, the hydrogen-bond network of the O4 channel becomes disconnected from the OEC. Additionally, major changes are observed in the W26, W27 and W28 positions of the water wheel region (the pentagon of waters W26–W30) in the O1 channel. The electron density of these waters become weaker in the S_2 state, suggesting higher mobility. The distances between these waters (W26–W27 and W26–W28) decreased to ~ 2.3 – 2.4 Å, the distance range consistent with shared protons between them.

In the S_2 to S_3 transition, a significant shortening of the D61–W1 distance occurs (2.78 to 2.51 Å), while the changes in this region are negligible between the S_1 and S_2 states. The short distance of the D61–W1 indicates a strong hydrogen bond with a shared proton between them (feature C). This transition involves a proton release to the lumen, and a Cl1 channel pathway from W1 via D61 has been proposed in the previous studies^{14,39}. Recent theoretical studies also support these observations^{40,41}. Our current observation supports a role of the Cl1 channel for the release of proton in the S_2 to S_3 transition. In contrast, the O4 channel, another potential proton release pathway^{42,43}, remains disconnected, as evidenced by the absence of a signal for W20 in the $2F_O - F_C$ data. The W26–W28 position of the water wheel region is well-defined again in the S_3 state, while the distances remain short.

Discussion

Knowledge of the S_3 state structure is a prerequisite to determine the mechanism of the water oxidation reaction in PS II. The key observations in this study support the O_X -inserted S_3 structure as discussed below. Based on these results, we illustrate experimentally observed structural changes during the S_1 – S_3 states at the OEC and its environment in Fig. 5, and discuss the hypothesis of the sequence of changes.

Structure of the S_3 state

The XFEL structural studies published by us and other authors in the recent years showed the insertion of a new ligand (O_X or O_6) into the cluster in the S_3 state. However, recent reports^{26,44,45} interpreted these XFEL data differently and proposed there is no binding of O_X in the S_3 state. The experiments in this study present a combination of higher resolution and anomalous diffraction data that provide crucial evidence for the binding of a new ligand in the S_3 state, and support the O_X -insertion model. The evidence for O_X includes (a) appearance of a 6.5σ positive difference electron density ($F_O - F_C$) peak in the

coordination sphere of Mn1 and Ca, (b) an 8.5σ omit electron density ($F_O - F_C$) peak for O_X , and (c) the $2F_O - F_C$ map features in the vicinity of Mn1, Ca and O5 showing a distinct secondary peak. In addition, (d) anomalous diffraction data collected at two different X-ray energies (7.0 keV and 9.5 keV) show expansion of the Mn_4Ca cluster upon application of two laser flashes to PS II, indicative of the insertion of O_X . No assumptions were made during the refinement regarding the oxidation states of the Mn, including in the formulation of restraints parameters. Therefore, the signal for O_X observed in the electron density maps is not the result of imposing any specific oxidation states.

The O_X -inserted S_3 structural model presented here was validated by testing different restraint conditions. We confirmed that the harmonic restraints (HR) used in this study are suitable for modeling the OEC geometry and showed that the O_X signal is not a manifestation of model bias. We also explored if a single-conformer model with no O_X could fit the observed electron density, although this would contradict independent spectroscopic evidence (MIMS, EPR, Mn K β XES)¹⁰. No evidence of an alternative O5 position was observed in this case, even when starting from different O5 positions with no Mn–O5 restraints (Supplementary Note 4 and Supplementary Fig. 10). In all cases, a substantial $F_O - F_C$ difference electron density ($> 5\sigma$) remains in the coordination sphere of Mn1/Ca, that can only be described by the presence of a new ligand in the OEC. In this context, the possibility of alternative conformers of D1:E189 in the vicinity of O_X was also tested, but the residue reverted back to the current S_3 conformation after refinement.

The characteristics of a long Mn4–O5 distance (2.2 Å)^{10,12–14,38} throughout the S-states has been discussed in the literature²⁸. O5 forms a distinct μ_3 -oxo-bridge, connecting Mn3, Mn4, and Ca, a feature that is maintained through the S-states. The oxidation states of Mn3 and Mn4 in the S_1 state are considered as Mn(IV) and Mn(III), respectively. Mn4 is most likely oxidized during the S_1 to S_2 transition to become +IV^{46,47}. Our data supports this observation, as Mn4 is the only Mn site that shows substantial changes in coordination distance during this transition (Fig. 4). However, the observed Mn4–O5 distance (2.22 Å in S_2 and S_3) is considered too long for the typical Mn(IV)–O_{bridge} interaction. DFT calculation of the OEC predicts it to be around 1.9 Å in both the S_2 and S_3 states²⁸. Synthetic Mn(IV)₃CaO₄ cubane and related complexes also show Mn–O distances around 1.8–1.9 Å^{48,49}.

Our current high-resolution data confirmed the longer Mn4–O5 distance in the S_1 – S_3 states. We speculate that this could be attributed to the distinct μ_3 -oxo character and a weakly bound Ca^{2+} ion in the cluster, giving O5, formally an oxo (O^{2-}) ligand, some hydroxo-like character (see also⁵⁰). Another reason discussed for such elongation of metal–ligand distances is radiation-induced reduction of the metal centers, a frequent artifact in synchrotron experiments⁵¹. However, this is not a concern in XFEL experiments, where ultrashort, intense X-ray pulses outrun such secondary damage processes^{52–54}. We confirmed the absence of radiation damage under our experimental conditions using in situ X-ray emission spectroscopy (XES)⁵⁵; the XES data show the normal advancement of the S-state cycle, including the expected Mn oxidation during the $S_1 \rightarrow S_3$ transitions and reduction during the $S_3 \rightarrow S_0$ transition^{10,55,56}.

Nature of O_X and the O5– O_X interaction

The improved resolution of the S_3 state data helped clarify the nature of O_X and the O5– O_X interaction. The Ca– O_X distance in the current study is 2.68 Å (± 0.09 Å) and Mn1– O_X distance is 1.75 Å (± 0.10 Å) which are consistent with previous measurements^{10,15}. Thus, both the Ca– O_X and Mn1– O_X distances confirm that O_X is a bridging ligand between Mn1 and Ca in the S_3 state.

The O5– O_X trace in the 2F data (Fig. 3c, left plot) exhibits a distinct but broad peak ranging from 2.0 to 2.3 Å near the O_X position. We find no evidence of the presence of a peroxide bond (1.4–1.5 Å) between O5– O_X in the S_3 state, as has been proposed^{12,57–59} or an oxo-oxyl

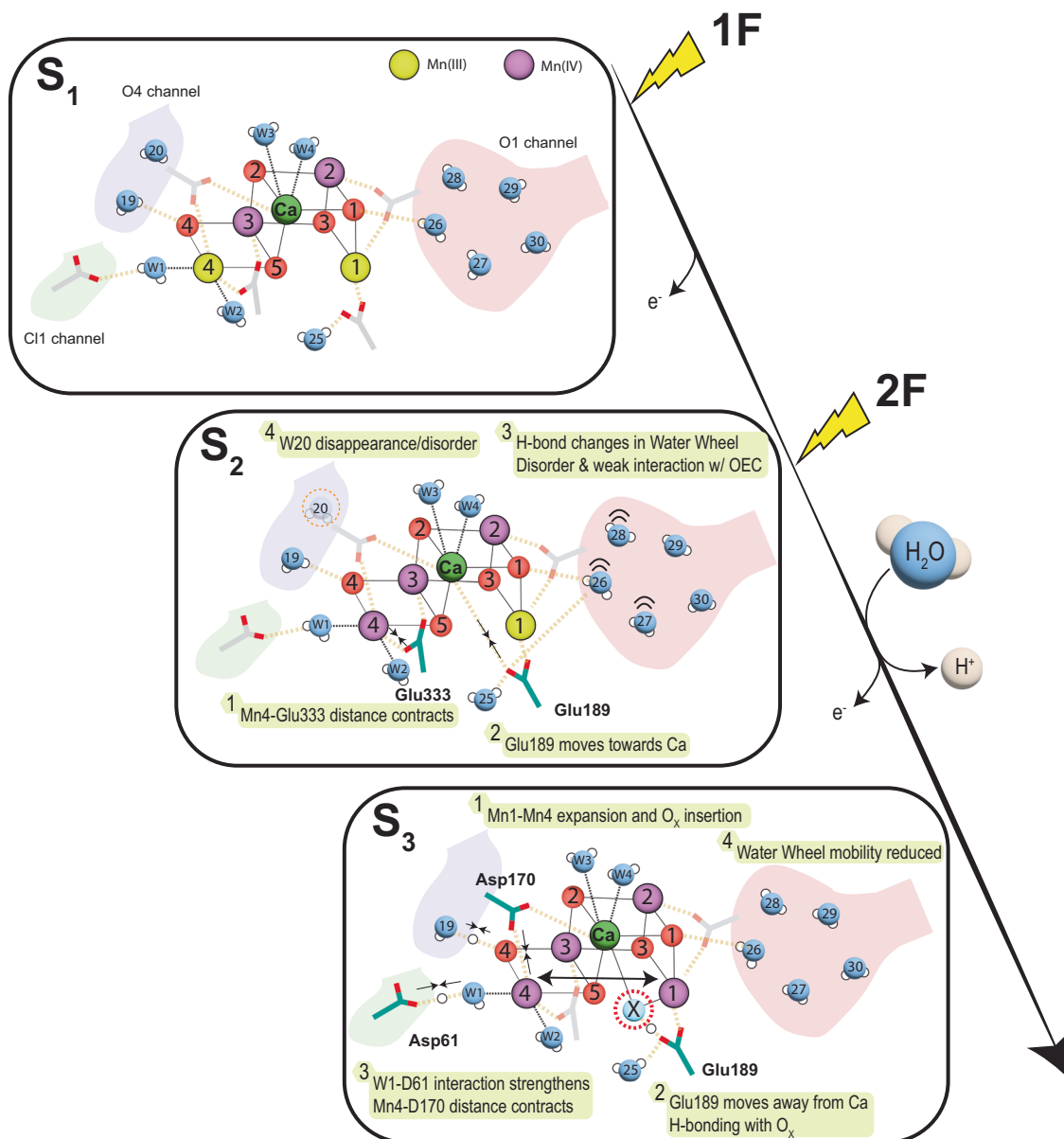


Fig. 5 | Summary of the changes in the S₁, S₂ and S₃ states near the OEC. The most important observation is the insertion of a new ligand, O_x (OH) bridging Mn1 and Ca in the S₃ state with expansion of the OEC (shown in red dashed circle). Significant changes in the Mn and Ca coordination environment are also observed. Modifications in the water/H-bond network indicate protonation rearrangements.

Mn atoms are shown in yellow (Mn(III)) and purple (Mn(IV)) while Ca and bridging oxygens in the cluster are green and red, respectively. Water molecules, including W1–W4 are shown as blue spheres with hypothesized proton positions shown as white spheres. Residues that change the most in a certain state are colored in teal while the rest are in light gray.

interaction (1.9 Å^{13,16}). In our current S₃ structure, O_x refined to a distance of 2.06 Å (±0.11 Å) from O5. This is similar to the geometry modeled in our previous work^{10,14}. Previous QM calculations have proposed an O5–O_x distance of 2.2–2.5 Å assuming a protonated O_x^{24,50}. The close distance between the D1:E189 and O_x (2.5 Å) points to the presence of a shared proton (Fig. 5), making it likely that O_x is in the OH[−] state.

Water network and protonation state changes near the OEC

The improved resolution of the S-states data offers an enhanced understanding of changes in the water network and possible protonation patterns. The S₁ to S₂ transition occurs with an oxidation of Mn (likely Mn4 from formally 3+ to 4+) without the release of a proton^{34,60–63}. A possible mechanism to compensate for the positive charge at the OEC in the S₂ state is the relocation of a proton from the Mn₄Ca cluster to a nearby region. Several groups proposed that the

D61–W1 region could serve for this purpose, by sharing a proton between them. Our data, however, did not show notable changes in this area in the S₁ to S₂ transition. In contrast, there are two regions that are changing; one is the water wheel region and the other is the W20 region. In the water wheel region, we observe shorter W28–W27 and W27–W26 distances accompanied by increased mobility of waters in the S₂ state. Such changes in the mode of interaction between the OEC and the water wheel region through O1 and W26 may compensate excess charges during the transitions. In the W20 region that is proximal to O4 in the OEC via W19, W20 disappears or becomes highly disordered in the S₂ state. This disconnects the H-bond network in the O4 channel for proton egress in the subsequent S-state transitions until it is restored in the S₀ state. These structural changes could suggest the excess proton is parked somewhere in the O4 channel or in the vicinity of the water wheel (Fig. 5).

In the S_3 state, the overall interaction between the OEC and the water wheel region remains the same as that in the S_2 state, but with better refined water positions of W26–28. A clear difference is seen in the D61 and W1 interaction that forms a strong H-bond interaction, possibly with a shared proton. This has important implications for the subsequent S_3 – S_0 transition where the first proton release step via D61 to the Cl1 channel is postulated to take several hundred microseconds^{15,64–66}.

During charge transfer between Yz and the OEC, as well as subsequent chemical changes at the OEC, Glu189 likely plays key roles through the S-state transitions. Glu189 is proximal to Yz/His190 via the backbone and forms a bridge between the water wheel (W26–W30) and Yz (via W25). Therefore, Glu189 probably senses the hole transfer in the early stage of the S-state transition, to change its chemical configuration. The 1st oxygen of Glu189 is always bound to Mn1 in all the S-states, while the 2nd oxygen changes its mode of interaction; in the S_1 state, Glu189 is a monodentate ligand, stabilized by surrounding water (W25, W26). In the S_2 state, this oxygen is bound to Ca to form a bidentate connection with Mn1 and Ca. In the S_3 state, Glu189 goes back to monodentate form, in which the 2nd oxygen is stabilized by H-bonding to the new ligand O_x (OH). We think this coordination mode change (monodentate vs. bidentate) of Glu189 is determined by the charge distribution between two electronegative oxygen atoms influenced by multiple parameters such as the oxidation state of Mn1 ligated to the 1st carboxylate oxygen, and energetic competition for the 2nd carboxylate oxygen between the Ca ion and the water network. The motion of Glu189 thus give us atomistic insights into the finely tuned dynamics utilized by biological catalysts.

In conclusion, in this study, we resolved existing ambiguities in the stable intermediate S-state structures, particularly in the S_3 state, by utilizing higher-resolution data to precisely determine Mn positions through anomalous density analysis. The anomalous density maps show well-defined metal positions, offering insight into the coordination environment of each metal in the catalytic site. Our data confirmed the presence of the new ligand (O_x/O6) in the S_3 state, bridging Mn1 and Ca. The results set the stage for the study of the subsequent S_3 to S_0 transition where the O–O bond is formed. In addition, the refinement workflow presented here provides a robust framework for analyzing serial crystallography data of metalloenzymes, effectively overcoming common challenges like limited resolution inherent to data collected under functional conditions.

Methods

Sample preparation

For the XRD measurements, 20–40 μm microseeding crystals of PS II dimers isolated from *Thermosynechococcus vestitus* (previously named, *Thermosynechococcus elongatus*) were obtained by using a modified microseeding protocol from⁶⁷. PS II microcrystals were grown in a buffer containing 100 mM Tris, pH 7.5, 100 mM ammonium sulfate and 15 % (w/v) PEG 5000MME. Following the post-crystallization protocol as described in ref. 68, PS II microcrystals were gradually transferred into a buffer with 100 mM Tris, pH 7.5, 100 mM ammonium sulfate and 35 % (w/v) as a final PEG concentration. To obtain the maximum oxygen activity, PS II microcrystal suspensions were exchanged from a Tris buffer at pH 7.5 to a final MES buffer at pH 6.5, 100 mM ammonium chloride and 35% (w/v) PEG 5000MME. PS II crystal suspension, at ~0.5–1.2 mM chlorophyll concentration, was loaded into a syringe (Hamilton gastight syringe, 1 ml) and dark-adapted for 1 h before data collection. Membrane inlet mass spectroscopy was used to determine the O₂ evolution, turnover parameters and S-state populations as described previously¹⁰. The PS II crystals showed no Mn (II) contamination based on XES and EPR measurements and exhibited an activity of $2500 \pm 100 \mu\text{mol O}_2/(\text{mg}(\text{Chl}) \times \text{h})$.

Sample injection and illumination

Acoustic droplet ejection (ADE) was used in combination with the Drop-on-Tape (DOT) sample delivery method⁶⁹. For capturing the stable intermediates S_2 , S_3 , each droplet of the crystal suspension was illuminated by 120 ns laser pulses at 527 nm using a Nd:YLF laser (Evolution, Coherent) at LCLS via three fiber-coupled outputs with a delay time of 200 ms between each illumination, and of 200 ms between the last illumination and the X-ray probe, similar to what was used previously in order to accommodate the acceptor quinone Q_A and Q_B kinetics, and efficiently drive S-state transitions. We implemented a feedback control system of the belt speed and deposition delay, and the flashing delay and droplet phase were adjusted accordingly. At the XFELs, a light intensity of $120 \pm 10 \text{ mJ}/\text{cm}^2$ was applied as O₂ evolution was found to be saturated at $70 \text{ mJ}/\text{cm}^2$ for the dimensions and concentrations of samples used in our experiments⁹.

X-ray data collection

X-ray diffraction (XRD) data was collected at the MFX endstation at LCLS (SLAC, Menlo Park, USA)⁷⁰ as part of proposals L1013621, L1019822, and L1035323. PS II crystals were measured with a beam energy of 9.5 keV, pulse energy of 1.5 mJ and pulse length of 35 fs. The X-ray spot size was $2.5 \mu\text{m} \times 2.5 \mu\text{m}$ (FWHM), determined based on a wire scan. Measurements were also taken with a 7 keV beam energy, the average pulse energy was 1.5 mJ, pulse length was 35 fs and the X-ray spot size $2.5 \mu\text{m} \times 2.5 \mu\text{m}$ (FWHM). The data was collected on the Rayonix MX-340HS detector used in a 3×3 binning mode (20 Hz data collection). This mode allows for collecting a sufficient number of images while maintaining enough spot separation between adjacent Bragg spots.

X-ray diffraction data processing

The data collected for the different illumination states were processed using the program *dials.stills.process*^{71,72} with a target unit cell of $a = 117.0 \text{ \AA}$, $b = 221.0 \text{ \AA}$, $c = 309.0 \text{ \AA}$, $\alpha = \beta = \gamma = 90^\circ$ and the space group $P2_12_12_1$. Bragg spots were integrated to the corners of the detector. Prior to integration, we also performed ensemble refinement of the crystal and detector parameters using the program *cctbx.xfel.stripe_experiment* which has been shown to narrow the unit cell distribution and improve the final isomorphous difference maps. Finally, the intensities were merged using the program *cctbx.xfel.merge* which applies a per-image resolution cutoff based on spot signal-to-noise and filtering of the lattices using a unit cell threshold of 1% from the reference model^{73,74}. The errors on the merged intensities were estimated using the MM24 error model⁷⁵ that uses concepts from robust statistics to reliably calibrate non-normal intensity statistics and outlier observations. No absorption correction from the kapton tape was applied in this work as the error is minimal compared to Bragg spot partiality and thus the correction does not lead to any appreciable improvement in data quality. During preliminary merging, we observed the skew of the unscaled signal-to-noise ($I/\sigma(I)$) showed an unusually high value in certain resolution bins. Theoretically, the skew is expected to go down monotonically toward zero at progressively higher resolutions (as the highest-angle bins, beyond the diffraction limits of the crystal sample, capture only Gaussian noise with an unskewed Normal distribution). Further investigation showed that in a few images ($< 0.1\%$), certain pixels had very high intensity count, possibly related to detector readout. These reflections were removed prior to scaling and merging, by implementing an additional reflection filter to reject Bragg spot outliers that particularly improves data in higher resolution bins (better than $2.5 \text{ \AA}$). The filter uses the isolation forest algorithm in the sklearn python library and is available in the filter step of *cctbx.xfel.merge* (using the *isolation_forest* parameter). We merged the data to the same resolution in 2 ways—by keeping the anomalous intensity pairs i.e. $I_{\text{obs}}(+)$, $I_{\text{obs}}(-)$, separate (called anomalous intensities in this

paper) as well as averaging them (called conventional intensities). The final unit cells and number of lattices merged for each dataset are tabulated in Supplementary Table 1 and 2.

Model building and refinement

Each dataset was refined in multiple steps using the workflow shown in Supplementary Fig. 7. The high resolution PS II structure (1.89 Å) published in a previous work (PDB ID: 7RF1) was used as the starting point for refinement using the program *phenix.refine*⁷⁶. The B-factors of all the atoms were set to 30 and the OEC (oxygen evolving complex) atoms and waters were removed from the starting model. In our starting model, we also changed the Phe41 residue in chain H/h (encoded by gene *PsbH*) to Cys based on our sequencing results. The initial refinement involved rigid body refinement to fit it into the unit cell, and subsequently tandem reciprocal space coordinate refinement and isotropic B-factor using the anomalous intensities. We used custom bonding restraints for chlorophyll-a (CLA, to allow correct placement of the Mg relative to the plane of the chlorin ring), and unknown lipid-like ligands (STE) in the refinement. By analyzing the anomalous diffraction maps, we placed the Mn and Ca in the centroid positions of each peak in the map. The model was then refined without any Mn-Mn or Mn-Ca restraints. We used custom bonding restraints for the amino acids coordinating the respective Mn/Ca atoms with a very loose set of restraints ($\sigma = 0.50$ Å, see Supplementary Data 2, item a). These custom restraints were used for the rest of the refinement workflow. We also refined the dispersive and anomalous scattering coefficients (f' , f'') for the Mn, Ca and Fe atoms with each element treated as one group in *phenix.refine*. In the next step, in order to improve the phases of the anomalous diffraction map, we added the bridging oxygens (and coordinating waters W1–W4) of the OEC in the center of the $F_o - F_c$ map density peaks and refined the model using two parallel strategies to evaluate the dependence of restraints (steps 3a, 3b in Supplementary Fig. 7). In strategy 3a, only Mn-O/Ca-O harmonic restraints (HR) were used with $\sigma = 0.05$ Å with the mean distances defined from previous OF structure¹⁰. The Mn-Mn distances were unrestrained in this step. In strategy 3b, done to validate the harmonic restraints, we used a flat-bottom harmonic restraints (FBHR, see profile in Supplementary Fig. 13 and implementation details in Supplementary Methods) with the region of 1.6–2.3 Å having no penalty for Mn-O coordination and then a harmonic penalty at either ends with $\sigma = 0.05$ Å. This was done in *phenix.refine* using an edits file (Provided in Supplementary Data 4) where we can provide a slack term to define the top hat region. The results of these 2 strategies are summarized in Supplementary Table 3 for all 3 flash states. The validation results showed very good overall agreement for the two strategies (RMS deviation ~ 0.05 Å over all OEC bond distances), leading us to use the refinement from strategy 3a (using Mn-O/Ca-O harmonic restraints). For OF and 1F datasets, we moved next to using conventional intensities and tried different strategies to evaluate the dependence of the restraints. In strategy 4a, we used the same Mn-O restraints (from 3a) and tightly restrained the Mn-Mn distances ($\sigma = 0.01$ Å) to the distances obtained from anomalous refinement in strategy 3a. This way we could incorporate the information from anomalous refinement into our mean intensity-based refinement. Strategy 4b used the FBHR restraints, similar to strategy 3b. Strategy 4c used the restraints from our previous publication¹⁴. The results from the three strategies are shown in Supplementary Table 4. We saw very good agreement for Mn-O distances in all 3 cases and no evidence for bias induced by the HR restraints. The results from strategy 4a were used for the final published model in this paper. The results from strategy 4c showed good agreement for the Mn-O and Mn-Mn distances (RMSD = 0.02 Å), underscoring our previously published restraints do an adequate job of modeling the OEC.

For the 2F dataset, an extra step (3a') involving the splitting of the model into 2 components was needed since the turnover in PS II is not

100% efficient resulting in about 60–75% S_3 population (from previous spectroscopy and EPR measurements¹⁰). In our single component refinement of the 2F data in step 3 (a, b), when we turned on water picking in *phenix.refine*⁷⁶ using default parameters for adding ordered solvent, a water (O_x) was automatically added to the Mn_4Ca cluster bridging Mn1 and Ca1 and about 2.2 Å from O5. No such water was added for the OF and 1F case. We further refined this water's occupancy, and also defined custom bonding restraints with Mn1/Ca/O5 to avoid non-bonded clashes in *phenix.refine*. We obtained the occupancy to be $\sim 65\%$ (See Supplementary Note 6 for further details). In accordance with this occupancy, the 2F model was split into a 2-component model in the active site region around the OEC - 65% S_3 (conformer B) and 35% S_2 (conformer A). The occupancy of the S_2 and S_3 components (including O_x) were kept fixed in the 2-component model. The 2-component region included portions of chains A/a (residues 54–66, 159–191, 296–300, 327–344 and the OEC), chains C/c (residues 327–329, 353–359) and chains D/d (residues 311–318, 351–352). The S_2 portion was taken from the 1F refined model and its coordinates were kept fixed and only a group B-factor adjustment (group_adp strategy in *phenix.refine*) was done. For the S_3 component, reciprocal space xyz refinement + individual isotropic B-factor refinement was done. The refinement here was first done using anomalous intensities with Mn-O restraints (step 3a'). We then did steps 4 (a, b, c, d) as detailed above. All waters (except the OEC coordinating W1–W4) were treated as a single component in this paper. For the 2F modeling, we also did a scan of different X-ray data weightings (wxc term in *phenix.refine*), from 0.1 (X-ray data weighted less) to 10.0 (X-ray data weighted more) and observed no significant change in the O_x geometry (RMS of bond distance changes is 0.1 Å between the two extreme cases) or the OEC. We started seeing evidence for overfitting ($R_{free} - R_{work} > 5\%$) for wxc values ~ 7 and determined that the automatic weighting optimization settings in *phenix.refine* provides the optimal R_{free} value (wxc ~ 2.5). These settings were used for the final 2F model. For the 7 keV anomalous datasets shown in this paper (Fig. 2), we used the method in strategy 1, 2, 3a for the data evaluation. In Supplementary Note 5, we show the effect of simultaneously refining the S_2 coordinates in the 2F model instead of keeping them fixed. The analysis justifies our strategy of keeping the S_2 coordinates fixed and also highlights factors to consider when modeling multi-component datasets. Our approach is further supported by the fact that the resolution of the 1F and 2F datasets are similar.

Structural data visualization

Manual model building and visualization was performed in Coot⁷⁷ and figures were generated using PyMol (version 3.14.1)⁷⁸. Raw diffraction images during data collection and reduction were visualized using the *dials.image_viewer* program in the *cctbx.xfel* package.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB) under PDB codes 9Z33 (OF, anomalous, 9.5 keV), 9Z34 (1F, anomalous, 9.5 keV), 9Z35 (2F, anomalous, 9.5 keV), 9Z36 (OF, anomalous, 7 keV), 9Z37 (2F, anomalous, 7 keV), 9Z68 (OF, conventional, 9.5 keV), 9Z69 (1F, conventional, 9.5 keV), 9Z6A (2F, conventional, 9.5 keV). Data used in this study from previously published datasets are available in the PDB under codes 6DHF (1F dataset at 2.08 Å) and 7RF1 (average S-state structure of PS II). Restraints and edits parameters used in *phenix.refine* for each of the dataset have been deposited in Zenodo as listed in Supplementary Table 11 and the DOIs are also available in the PDB mmCIF file under the category *_pdbx_related_exp_data_set.data_reference*. The raw X-ray free-

electron laser diffraction images have been deposited in the Coherent X-Ray Imaging Database, www.cxidb.org (ID 246) with a DOI: 10.11577/3366809. Source data for Figs. 2c and 3c and Supplementary Figs. 2c, 3c, 5, 8, 10b, 12, 13, 14 and 18 are provided as a Source Data file. Source Data are provided as a Source Data file. Source data are provided with this paper.

Code availability

The open-source programs *dials.stills.process*, the *cctbx.xfel* GUI and *cctbx.xfel.merge* are distributed with DIALS packages available at <http://dials.github.io>, with further documentation available at <http://ccilbl.gov/xfel>. Instructions are provided in the CXIDB entry 246 for processing the raw diffraction data with *cctbx.xfel*. All structural refinement were done using the *phenix.refine* program as part of phenix-2.0 development version. Custom code developed for the results shown in this paper is available at <https://github.com/asmit3/eden>⁷⁹ with instructions therein to run these scripts.

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Competing interests

The authors declare no competing interests.

Additional information

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