nature portfolio

Peer Review File

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

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Bhowmick and coworkers describes structural studies on the photosynthetic complex photosystem II, the site for water oxidation in plants and cyanobacteria. Many structures have been published for not only the dark S1 state but the different S-states that the complex undergoes during the conversion of water to molecular oxygen. The paper presents new X-ray diffraction data, combining conventional measurements with anomalous measurements to focus on the structural changes that occur at the active site, a Mn4CaO5 metal center, during the water oxidation reaction. The authors detail how the incorporation of anomalous data coupled with careful consideration of the refinement yields a more accurate model for the intermediate S-states, especially the status of oxygen atoms and their bonding rearrangements with the Mn-cluster. The resulting maps are analyzed very thoroughly and carefully. The consideration of the restraints and refinement process coupled with detailed information is a welcome inclusion to such structural studies. Their arguments concerning the positions of oxygen atoms in the S3 state are convincing and I fully support the publication of this manuscript, with only minor additional considerations in the discussion:
Although the paper emphasizes the S3 state, the manuscript includes the determination of the structures of the S1 and S2 states. The authors should include a more complete discussion of these states with a comparison of the new models to previously published models.
While the strong focus on the structural analysis is clearly useful, the authors could broaden the appeal of this work to a broader Nature Communications audience by including a discussion of the implications of these outcomes on other experimental information about the Mn-cluster and the protein environment, including QM/MM with citation of a recent paper in JACS (2026, 148, 9255).
There are various improper references in the text, for example page 19 (Nature 2018), page 20 (Kern 2018), and page 24 (Nature 2018/PNAS 2020).

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
This manuscript presents high-resolution (~1.9 Å) XFEL crystallographic data addressing the presence of an additional oxygen ligand (OX/O6) in the S_3 state of Photosystem II. The combination of anomalous diffraction and improved conventional data is a clear technical advance, and the authors provide multiple lines of evidence supporting OX insertion. The effort to examine restraint dependence is also exemplary and goes far beyond typical crystallographic analysis.

However, several issues limit the strength of the central conclusion:

1. The key 2F dataset represents a mixed population, yet the refinement treats the S_2 component as fixed (refining only ADPs) while refining only the S_3 component. This asymmetric treatment could introduce bias towards the S_3 model and may artificially stabilize the OX assignment. Why was this strategy chosen?
While fixing a well-defined minority component can be a pragmatic choice in mixed-state refinement, it would be important to demonstrate that allowing limited coordinate freedom for the S_2 component (e.g. with tight restraints) does not materially

affect the OX feature.

2. The identification of OX relies on model-phased maps in a region adjacent to Mn ions, where Fourier artefacts and peak shifts are expected. While the authors provide omit-map and profile-based analyses, these do not fully establish that the density uniquely requires an additional ligand rather than reflecting alternative interpretations (e.g. disorder or mixed occupancy). The omit maps partially mitigate model bias, but still rely on phases from the surrounding model. A more stringent test would involve, for example, simulated-annealing composite omit maps or comparison to phases derived independently (e.g. from anomalous signal).

3. While the authors examine the effect of different restraint schemes, all refinements assume the same coordination topology (including the presence of OX). As a result, the analysis does not test whether the data themselves require this topology and the conclusion that the model is independent of restraints should be stated more cautiously. The single-conformer analysis (SI Fig. 10 and associated discussion), which explores refinements without OX, is an important step in this direction. However, it represents only one alternative model and does not fully explore the range of possible topologies or occupancy scenarios. The point remains that the robustness of the OX assignment with respect to model choice is not yet fully established.

4. The manuscript employs END/RAPID-style synthetic datasets to propagate uncertainties in geometric parameters of the OEC. However, this analysis is performed within a fixed structural model that includes OX, and does not assess whether the OX feature itself is robust under resampling of the diffraction data. Extending the resampling analysis to evaluate the reproducibility of the OX density (e.g. peak presence, occupancy and distances) would substantially strengthen the claim that OX is required by the data. Since this is serial crystallography data, I would suggest that the authors consider bootstrapping approaches as described by Grünbein and Gorel et al. (https://pmc.ncbi.nlm.nih.gov/articles/PMC7960726/) as an alternative or complement to the END/RAPID analysis. It should be noted that such resampling would test the reproducibility of the feature at the data-merging level and would therefore complement, rather than replace, the analysis of model bias discussed above.

Recent work (Wang, https://pmc.ncbi.nlm.nih.gov/articles/PMC11912364/) highlights the sensitivity of OEC ligand assignment to model bias and mixed-state heterogeneity and argues that features interpreted as O6 can arise from refinement assumptions. While the present manuscript uses a broader set of evidence than that analysis, these considerations reinforce the need to demonstrate that the OX assignment is robust to alternative modelling choices.

Finally, the reported OX occupancy is indirect and lacks uncertainty estimates and mechanistic conclusions regarding O–O bond formation appear somewhat stronger than justified by the data.

Overall, this is an important study and I recommend major revision, with particular emphasis on demonstrating that the OX assignment is robust with respect to multi-state modelling, refinement assumptions and data resampling.

Minor points:

• A comparison of local B-factors for OX relative to neighboring atoms (especially O5 and nearby waters) would strengthen the interpretation, in particular given the partial occupancy.
• The manuscript states that the X-ray term is weighted more strongly than the restraint term, but it is unclear how sensitive the refined geometry (especially OX position) is to this weighting. Some clarification would be useful.
• The occupancy of OX is discussed, but it is not entirely clear how strongly it is constrained during refinement. A short clarification of the refinement strategy (fixed vs refined occupancy, restraints, correlations with B-factors) would improve transparency.
• P 13 line 371-372: please cite the reports which interpreted XFEL data differently.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
While much has been learned about the structure and function of Photosystem II (PSII), the complete chemical mechanism of biological water oxidation remains elusive. Elucidating this mechanism requires detailed knowledge of the structure of the oxygen-evolving complex (OEC) in its multiple redox intermediates. Determining these structures is notoriously challenging, and many of the authors of the present manuscript have contributed influential studies and techniques to solve these challenges.

Currently, experimental XFEL structures of the OEC have been criticized for two primary reasons. First, metal-ligand bond distances and metal-metal distances are often longer than expected based on model complexes and quantum chemistry calculations. Second, the Mn ions are such strong scatterers that positioning O atoms in between them is difficult.

In this work, the authors address these challenges by first collecting data sets at two different X-ray energies. They used the resulting anomalous diffraction data to accurately determine only the positions of the Mn ions. The resulting Mn-Mn distances are generally quite reasonable. They then built atomic models restricted by these Mn positions that include oxo

ligands. At this step, I encourage the authors to relate the Mn-Mn distances determined in this structural study to those from EXAFS (preferably extended) measurements by themselves and others. Based on my comparisons, they seem to match well within 0.1 Angstrom. My only concern is the 3.4 Angstrom distance observed in the 2-flash data set. This comparison and discussion of any variation would be best in the main text of the manuscript.

With the resulting OEC structures, isomorphous difference maps were prepared comparing the difference flash states. In the 2-flash minus 1-flash analysis, obvious density in an area not occupied by a known oxo ligand appears. In a separate model-dependent analysis that is worth performing but not as convincing, Fo – Fc omit maps show that if a new Ox is modeled in the 2-flash data set, unaccounted for density remains at a site not occupied by a known oxo ligand when it is removed.

Based on the data presented, I encourage the authors to change their title to a statement, “An additional water is introduced into the manganese cluster during the formation of the S3 state of Photosystem II.” The data as presented support that it is there. Importantly, Ox is at a position greater than 2 Angstroms away from O5, so clearly there is no peroxo or other intermediate has formed after two flashes. This means that O-O bond formation happens later. That detail is a key finding that helps us take one more step towards a complete chemical mechanism of water oxidation.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors did a nice job addressing the various comments. I recommend publication.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
I would like to thank the authors for answering all my questions and for revising the manuscript. I think it is now in a state to be published.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have addressed the minimal corrections I requested. I remain enthusiastic about the important results provided by this work.

(Remarks on code availability)
N/A

We thank the reviewers for the detailed and very constructive comments they have provided. We have answered all the questions in detail, and a point-by-point response to the comments and the changes made in the manuscript are described below. We are listing below the reviewers' comments in black with our responses below them in blue. Changes made in the main text and SI have been highlighted in yellow.

Reviewers' comments:

Reviewer #1

1. The manuscript by Bhowmick and coworkers describes structural studies on the photosynthetic complex photosystem II, the site for water oxidation in plants and cyanobacteria. Many structures have been published for not only the dark S_1 state but the different S-states that the complex undergoes during the conversion of water to molecular oxygen. The paper presents new X-ray diffraction data, combining conventional measurements with anomalous measurements to focus on the structural changes that occur at the active site, a Mn_4CaO_5 metal center, during the water oxidation reaction. The authors detail how the incorporation of anomalous data coupled with careful consideration of the refinement yields a more accurate model for the intermediate S-states, especially the status of oxygen atoms and their bonding rearrangements with the Mn-cluster. The resulting maps are analyzed very thoroughly and carefully. The consideration of the restraints and refinement process coupled with detailed information is a welcome inclusion to such structural studies. Their arguments concerning the positions of oxygen atoms in the S_3 state are convincing and I fully support the publication of this manuscript, with only minor additional considerations in the discussion:

We thank the reviewer for the positive comments and for recommending publication of the manuscript. The additional points raised are addressed below.

2. Although the paper emphasizes the S_3 state, the manuscript includes the determination of the structures of the S_1 and S_2 states. The authors should include a more complete discussion of these states with a comparison of the new models to previously published models.

We agree with the reviewer that such a comparison is useful, and we have now added a section on Page 13 discussing the S_1 and S_2 states with a comparison with previous models.

3. While the strong focus on the structural analysis is clearly useful, the authors could broaden the appeal of this work to a broader Nature Communications audience by including a discussion of the implications of these outcomes on other experimental information about the Mn-cluster and the protein environment, including QM/MM with citation of a recent paper in JACS (2026, 148, 9255).

As suggested by the reviewer, we have now included the JACS paper reference (it was not available when the manuscript was initially submitted) in the revised version on Page 14. We have also now added relevant material connecting the results to previous EXAFS measurements (also requested by reviewer 3) on Page 8 and to other experimental information about the OEC. We note that there is already an extensive discussion in the manuscript on the mechanistic implication of

the results for the broader water oxidation/bioenergetics community. Based on the reviewer's suggestion, we have now highlighted in the Introduction (Page 6), the broad applicability of the analysis method to identify subtle changes in functional data of metalloenzymes catalysis (in the Introduction) that would be of interest to many groups in the field. We hope this will be of appeal to a broad readership.

4. There are various improper references in the text, for example page 19 (Nature 2018), page 20 (Kern 2018), and page 24 (Nature 2018/PNAS 2020)

We thank the reviewer for the careful reading of the manuscript and for pointing out the incorrect references, which have now been fixed.

Reviewer #2:

This manuscript presents high-resolution (~1.9 Å) XFEL crystallographic data addressing the presence of an additional oxygen ligand (O_X/O6) in the S_3 state of Photosystem II. The combination of anomalous diffraction and improved conventional data is a clear technical advance, and the authors provide multiple lines of evidence supporting O_X insertion. The effort to examine restraint dependence is also exemplary and goes far beyond typical crystallographic analysis.

We thank the reviewer for the constructive comments, and we address the other concerns raised in a point-by-point manner below.

However, several issues limit the strength of the central conclusion:

1. The key 2F dataset represents a mixed population, yet the refinement treats the S_2 component as fixed (refining only ADPs) while refining only the S_3 component. This asymmetric treatment could introduce bias towards the S_3 model and may artificially stabilize the O_X assignment. Why was this strategy chosen?

While fixing a well-defined minority component can be a pragmatic choice in mixed-state refinement, it would be important to demonstrate that allowing limited coordinate freedom for the S_2 component (e.g. with tight restraints) does not materially affect the O_X feature.

The reviewer brings up an important detail in the refinement pipeline. We have now added controls to justify the fixed S_2 strategy, please see Supplementary Discussion 5 and SI Figure 16 and these are mentioned on Page 22 in the Methods. We have added details as to why a fixed S_2 strategy was chosen (the reviewer him/herself suggests the predominant reason in the second point) in Supplementary Discussion 5 and SI Figure 16. As for the second point, we checked for the robustness of the O_X feature by simultaneously refining the S_2 and S_3 components using 2 approaches: (a) Refining the S_2 component with no limitation on coordinate movement, and (b) Refining S_2 with additional coordinate restraints using the 1F(S_2) reference model. As now highlighted in the Supplementary Discussion section 5, case (a) provides a much worse agreement for the S_2 component with the known 1F(S_2) structure and potentially introduces compensatory errors into the unknown S_3 structure (e.g., Ca-Mn distances). Several deviations are observed in coordination bond distances. On the other hand, in (b), limited freedom of the coordinates (as suggested by the reviewer) reproduces the S_2 geometry quite well. The S_3 geometry is also similar to the model presented in this paper (i.e., using a fixed S_2 component). In both cases, the O_X features are similar as shown in SI Figure 16. Based on the above evidence, we believe the best practice is to constrain or tightly restrain the S_2 coordinates during refinement of the 2F model. We also want to emphasize that a single component refinement against the 2F data also showed clear indication for the presence of an additional oxygen at the O_X position (SI Fig. 10).

2. The identification of O_X relies on model-phased maps in a region adjacent to Mn ions, where Fourier artefacts and peak shifts are expected. While the authors provide omit-map and profile-based analyses, these do not fully establish that the density uniquely requires an additional ligand rather than reflecting alternative interpretations (e.g. disorder or mixed occupancy). The omit maps partially mitigate model bias, but still rely on phases from the surrounding model. A more stringent

test would involve, for example, simulated-annealing composite omit maps or comparison to phases derived independently (e.g. from anomalous signal)

We thank the reviewer for the comment but would like to point out issues related to the types of maps suggested:

a) Simulated-annealing composite omit maps, while used for such contexts, are an aggressive-invasive technique and do not yield a unique answer. The annealing process is likely to wipe out subtle features such as the O_X density that we are examining here, as it degrades map quality substantially, while reducing bias. It also can cause the molecule to diverge out of density. After thorough discussion within our group, including researchers from the PHENIX software development team, we think this is not an appropriate technique for showing phase-insensitivity of O_X density. We propose an alternate solution using Polder omit maps below. For the purpose of this author response only, we calculated the simulated annealing $2F_O$-F_C composite omit map for the 2F state using default settings in the PHENIX software. This data is shown below (but is not included in the manuscript). The data shows very subtle changes in the 2F map near the O_X region but it is hard to conclude if it is due to an additional ligand. This is consistent with our experience with such maps for detecting weak features like O_X.

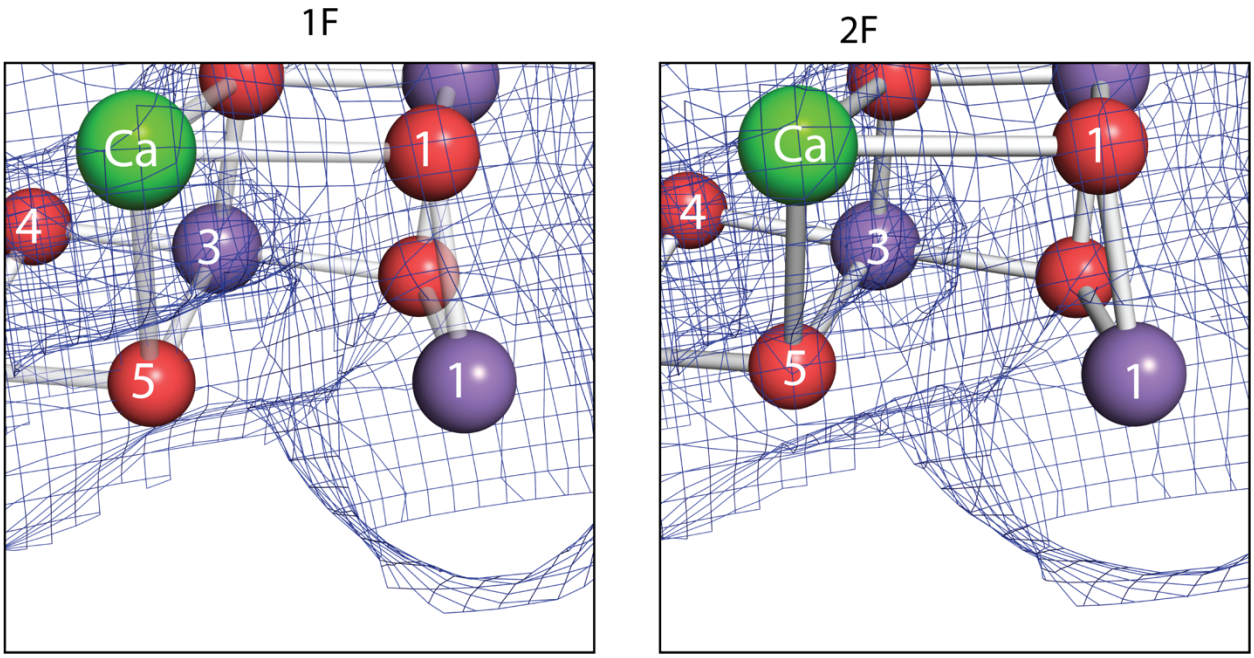

Figure Caption: Simulated Annealing $2F_O$-F_C composite omit map are shown at a 1.5σ level with the OEC cluster overlaid. The O_X atom is not shown in the 2F model for visual clarity.

b) Maps/phases from anomalous data are usually much lower in quality and are improved through iterative model building after initial identification of the heavy atom sub-structure. For a large complex like PS II (~350 kDa), the phasing power of the pure anomalous signal from 4 Mn atoms and 2 Fe atoms is very low in the current datasets. We thus do not consider this to be a viable method for O_X density identification.

In light of the reviewer's concerns, we have now calculated Polder F_O-F_C difference omit maps, with some modifications which is now described in the manuscript. We think Polder maps are a more appropriate tool for examining ligand density and are sensitive to features such as oxygen density. Polder maps also have the advantage of not including bulk solvent in the omitted regions, which would otherwise suppress the difference signal for O_X. We describe the results in SI Figure 17, and in the main text on Page 10 with the map calculation steps described in Supplementary Methods under Map Calculations. Before running the Polder omit map calculation, we ran a few pre-processing steps to remove phase memory of the active site. Briefly, we deleted all the 1st shell amino acids and waters as well as the Mn_4Ca cluster and re-refined the protein (after randomizing B-factors and xyz by a rms of 0.25 Å) for 100 cycles. The long refinement cycle after randomization ensures the memory of the initial phases is lost. Subsequently, we calculated the Polder omit map in this deleted region. The phases used now contain no information about the OEC or the surrounding shell. A distinct additional density is seen in the 2F map that is not visible in the 1F map, supporting insertion of the additional ligand, O_X.

3. While the authors examine the effect of different restraint schemes, all refinements assume the same coordination topology (including the presence of O_X). As a result, the analysis does not test whether the data themselves require this topology and the conclusion that the model is independent of restraints should be stated more cautiously. The single-conformer analysis (SI Fig. 10 and associated discussion), which explores refinements without O_X, is an important step in this direction. However, it represents only one alternative model and does not fully explore the range of possible topologies or occupancy scenarios. The point remains that the robustness of the O_X assignment with respect to model choice is not yet fully established.

While we appreciate the reviewer's comment, it is unclear how additional topologies could be explored for the OEC in the S_3 state beyond the scenarios presented in the manuscript (e.g., in SI Figure 10 and SI Figure 7). We note that at this point in time, the detailed structural studies of the S-states carried out by our group and others have narrowed the topologies to the ones discussed in the manuscript. A few points are highlighted below to clarify the reviewer's concern.

(a) The OEC model was built iteratively, first without any OEC atom, then with just Mn atoms (in the anomalous density), then the 5 bridging oxygen atoms (O1-O5) in the residual F_O-F_C map and finally the 6th oxygen, O_X, in the remaining F_O-F_C density. These steps are discussed in the paper (see Methods on Page 20-21) and shown in SI Figure 7. The model building thus used the data as the evidence to place the atoms with no assumed topology. The restraints are needed to ensure the atoms, especially O atoms, stay in density and the refinement does not diverge.

(b) In SI Figure 10, the refinements used Flat Bottom Harmonic Restraints (FBHR). This restraint type does not enforce any topology beyond Mn-O coordination bonds (that have no penalty between 1.6-2.3 Å). No angle restraints are used and restraints for the coordination bonds with the protein surrounding are very lax ($\sigma = 0.50\dot{A}$). The Mn positions were taken from the anomalous data. Thus, the OEC is free to adopt any topology provided it explains the 2F data. The starting OEC structures were also randomized to explore a large phase space. We have now updated the figure (new panel a) to better convey this information and emphasize that no topology was enforced.

(c) Much of the OEC topology in the S_1-S_3 states is determined by the protein pocket, including non-bonded contacts (e.g., Van der Waals repulsion). Thus, any new topology the OEC might hypothetically adopt in the S_3 state has to be consistent with the protein changes. These protein changes would be reflected in the isomorphous difference maps (Figure 3a and SI Figure 3a) which are minimally model biased, and which show evidence for O_X. After re-examination and deliberation of the data, we are unable to find possible alternative explanations beyond what is discussed in this paper.

4. The manuscript employs END/RAPID-style synthetic datasets to propagate uncertainties in geometric parameters of the OEC. However, this analysis is performed within a fixed structural model that includes O_X, and does not assess whether the O_X feature itself is robust under resampling of the diffraction data. Extending the resampling analysis to evaluate the reproducibility of the O_X density (e.g. peak presence, occupancy and distances) would substantially strengthen the claim that O_X is required by the data. Since this is serial crystallography data, I would suggest that the authors consider bootstrapping approaches as described by Grünbein and Gorel et al. (https://pmc.ncbi.nlm.nih.gov/articles/PMC7960726/) as an alternative or complement to the END/RAPID analysis. It should be noted that such resampling would test the reproducibility of the feature at the data-merging level and would therefore complement, rather than replace, the analysis of model bias discussed above.

We thank the reviewer for suggesting the innovative bootstrapping approach. We have now added a new error analysis section that uses this technique along with the END/RAPID analysis. New software in *cctbx.xfel.merge* was developed to calculate the resampled and remerged MTZ files along with a pipeline to run the 100 refinement trials. The procedure is now described in the Supplementary Methods section and the results are described in the Supplementary Discussion section 6. The calculations show that O_X density is robust to resampling of the diffraction data (average F_O-F_C omit map peak height ~ 6.0 +/- 0.5 σ) and occupancy refinement yields an O_X population of 0.64 +/- 0.07.

Recent work (Wang, https://pmc.ncbi.nlm.nih.gov/articles/PMC11912364/) highlights the sensitivity of OEC ligand assignment to model bias and mixed-state heterogeneity and argues that features interpreted as O6 can arise from refinement assumptions. While the present manuscript uses a broader set of evidence than that analysis, these considerations reinforce the need to demonstrate that the O_X assignment is robust to alternative modelling choices.

Finally, the reported O_X occupancy is indirect and lacks uncertainty estimates and mechanistic conclusions regarding O–O bond formation appear somewhat stronger than justified by the data.

Regarding the first point about O_X occupancy, this has been discussed by our group previously in Ibrahim et al., *PNAS*, 2020, using omit map analysis as well as X-ray emission spectroscopy data. To respond to the reviewer, we have now added a new section in the Supplementary Discussion section 6 and SI Figure 18 on the occupancy analysis of O_X. Two additional methods were used to determine O_X population in the crystallographic data - (a) Occupancy analysis of O_X within a single component S_3 model. (b) Refining a 2-component model with fixed occupancies (in the range of 0-100% S_3 in steps of 10% population) and tracking the Fo-Fc peak height at O_X. The optimal population would give a residual peak height ~ 0. Both approaches converge on a window

of 60-70% population for O_X. This is further supported by the bootstrapping approach which yields a population of 0.64 +/- 0.08, thus providing an additional error estimate on the occupancy.

We are actually somewhat unsure what the reviewer refers to regarding O-O bond formation. However, if the reviewer is referring to O-O bond formation in the S_3 state, then we respectfully disagree with the reviewer on the second point about conclusions regarding O-O bond formation. Detailed analysis of electron density maps (Figure 2b,c and SI Figure 2b,c) and error analysis (SI Table 6) have been presented in this paper to examine whether O-O bond formation is possible in the 2F data. Furthermore, refinement done with FBHR restraints (SI Table 3) also were done to check if any bias is introduced in the O positions that would prevent a short O-O distance in refinement. We also examined the penalty on the restraints (such as O5-O_X) and observed no substantial strain in the modelled position ($Z < 2.0$, SI Table 7). These calculations provide strong evidence that an O-O bond is not formed in the S_3 state. We thus stand by our mechanistic conclusions about no O-O bond being formed in the S_3 state.

Overall, this is an important study and I recommend major revision, with particular emphasis on demonstrating that the O_X assignment is robust with respect to multi-state modelling, refinement assumptions and data resampling.

We again thank the reviewer for the comments, and we hope our responses and the new evidence provided by the work suggested by the reviewer demonstrates the robustness of the O_X signal.

Minor points:

• A comparison of local B-factors for O_X relative to neighboring atoms (especially O5 and nearby waters) would strengthen the interpretation, in particular given the partial occupancy.

A new SI Table 8 has been added for this comparison and we mention this point in the main text on Page 10

• The manuscript states that the X-ray term is weighted more strongly than the restraint term, but it is unclear how sensitive the refined geometry (especially O_X position) is to this weighting. Some clarification would be useful.

We have now modified this statement and moved it to the appropriate location in the Methods section on Page 22 where the 2F modeling is described. We have also added some information regarding the sensitivity of the refined geometry to this weighting.

• The occupancy of O_X is discussed, but it is not entirely clear how strongly it is constrained during refinement. A short clarification of the refinement strategy (fixed vs refined occupancy, restraints, correlations with B-factors) would improve transparency.

A clarification of the refinement strategy has been provided now for occupancy of O_X in the Model building and refinement section under Methods (Page 22). In the refinement for the final 2F structure, the O_X population is kept fixed at 65%. B-factors and coordinates are allowed to refine.

• P 13 line 371-372: please cite the reports which interpreted XFEL data differently.

We thank the reviewer for this suggestion and this has now been done.

Reviewer #3:

While much has been learned about the structure and function of Photosystem II (PSII), the complete chemical mechanism of biological water oxidation remains elusive. Elucidating this mechanism requires detailed knowledge of the structure of the oxygen-evolving complex (OEC) in its multiple redox intermediates. Determining these structures is notoriously challenging, and many of the authors of the present manuscript have contributed influential studies and techniques to solve these challenges.

Currently, experimental XFEL structures of the OEC have been criticized for two primary reasons. First, metal-ligand bond distances and metal-metal distances are often longer than expected based on model complexes and quantum chemistry calculations. Second, the Mn ions are such strong scatterers that positioning O atoms in between them is difficult.

In this work, the authors address these challenges by first collecting data sets at two different X-ray energies. They used the resulting anomalous diffraction data to accurately determine only the positions of the Mn ions. The resulting Mn-Mn distances are generally quite reasonable. They then built atomic models restricted by these Mn positions that include oxo ligands.

1) At this step, I encourage the authors to relate the Mn-Mn distances determined in this structural study to those from EXAFS (preferably extended) measurements by themselves and others. Based on my comparisons, they seem to match well within 0.1 Angstrom. My only concern is the 3.4 Angstrom distance observed in the 2-flash data set. This comparison and discussion of any variation would be best in the main text of the manuscript.

We thank the reviewer for the comments. We have now added SI Table 9 with the different Mn-Mn distances from the XFEL structures for the S_1-S_3 states and comparison with EXAFS data from our group (Glockner et. al., JBC, 2013). The results are also discussed in the main text on Page 8.

2) With the resulting OEC structures, isomorphous difference maps were prepared comparing the difference flash states. In the 2-flash minus 1-flash analysis, obvious density in an area not occupied by a known oxo ligand appears. In a separate model-dependent analysis that is worth performing but not as convincing, Fo – Fc omit maps show that if a new O_X is modeled in the 2-flash data set, unaccounted for density remains at a site not occupied by a known oxo ligand when it is removed.

Based on the data presented, I encourage the authors to change their title to a statement, “An additional water is introduced into the manganese cluster during the formation of the S_3 state of Photosystem II.” The data as presented support that it is there. Importantly, Ox is at a position greater than 2 Angstroms away from O5, so clearly there is no peroxo or other intermediate has formed after two flashes. This means that O-O bond formation happens later. That detail is a key finding that helps us take one more step towards a complete chemical mechanism of water oxidation.

The title of the paper has now been modified, as suggested. We thank the reviewer for the encouraging review.