An oxyl/oxo mechanism for oxygen-oxygen coupling in PSII revealed by an x-ray free-electron laser

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Photosynthetic water oxidation is catalyzed by the Mn4CaO6 cluster of photosystem II (PSII) with linear progression through five S-state intermediates (S0 to S5). To reveal the mechanism of water oxidation, we analyzed structures of PSII in the S1, S2, and S3 states by x-ray free-electron laser serial crystallography. No insertion of water was found in S2, but flipping of D1 Glu348 upon transition to S2 leads to the opening of a water channel and provides a space for incorporation of an additional oxygen ligand, resulting in an open cubane Mn3CaO4 cluster with an oxyl/oxo bridge. Structural changes of PSII between the different S states reveal cooperative action of substrate water access, proton release, and dioxygen formation in photosynthetic water oxidation.

Photosynthetic water oxidation by plants, algae, and cyanobacteria converts light energy from the sun into chemical energy in the form of sugar and concurrently releases dioxygen into the atmosphere, thereby sustaining all aerobic life on Earth. The first reaction in oxygenic photosynthesis occurs in PSII, which harvests the oxygen-evolving complex (OEC) that catalyzes stepwise oxidation of water through the S-state cycle (S0 to S5). To reveal the mechanism of water oxidation, we analyzed structures of PSII in the S1, S2, and S3 states by x-ray free-electron laser serial crystallography. No insertion of water was found in S2, but flipping of D1 Glu348 upon transition to S2 leads to the opening of a water channel and provides a space for incorporation of an additional oxygen ligand, resulting in an open cubane Mn3CaO4 cluster with an oxyl/oxo bridge. Structural changes of PSII between the different S states reveal cooperative action of substrate water access, proton release, and dioxygen formation in photosynthetic water oxidation.

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Time-resolved, pump-probe x-ray free electron laser crystallographic analysis of PSII in the S3 state at 2.35-Å resolution showed an incorporation of an oxygen O6 into the OEC in the vicinity of O5, supporting a dioxygen formation mechanism between O5 and O6 (11). This reaction can proceed via either (i) an oxyl/oxo radical coupling (12), (ii) a nucleophilic attack reaction mechanism (13), or (iii) a peroxide intermediate mechanism (14). Owing to the limited resolution of the S3-state structure, however, the chemical entity of the oxo intermediates (superoxo, peroxo, oxyl/oxo, and hydroxo/oxo) has not been identified, therefore the reaction mechanism was not determined unambiguously.

The OEC is located at a node of five water channels (4) (Fig. 1) involved in proton release, balancing the net charge of the OEC, and inlet of substrate water (11, 15). Dislocation of water W665 in the O4 channel upon progress of the S3 state results in closure of the 15-Å-long, water-mediated hydrogen bonding network (11, 16). We previously attributed this structural change to a proton release through the O4 channel, whereas others have argued that W665 serves as the source of the O6 atom involved in O=O bond formation (17, 18). Lack of a high-resolution structure of the S2 state has contributed to uncertainty about the structural changes leading to or from this state. To address these issues, we fixed PSII in the S5, S2, and S1 states, and the triply flashed (3F) state by a cryo-trapping method with microcrystals of PSII from the thermophilic cyanobacterium *Thermosynechococcus vulcanus*. Structures at 2.15- to 2.50-Å resolution using fixed-target serial femtosecond crystallography (SFX) are consistent with the structures at room temperature determined previously (11, 16) but allow more accurate determination of interatomic distances in the OEC, revealing the chemical entity of the intermediate O5 and O6 species and the corresponding changes in the protein-ligand environment.

Fig. 1. Water networks in PSII.
Five hydrogen-bonded water networks surround the OEC (PDB ID 4UB6) (5). The upper-bound distance for the hydrogen bonds shown is 3.3 Å. CL1 and CL2 represent chloride ions found near the OEC.
Fig. 2. Structural changes of the OEC during the S$_i$-state transitions. (A to C) OEC structures superimposed with $F_{\text{obs}} - F_{\text{calc}}$ isomorphous difference Fourier maps of (A) 1F minus dark, (B) 2F minus 1F, and (C) 2F minus dark datasets. Structures before and after $S_i$-state transition are shown in gray and color, respectively. Difference maps are contoured at $-3\sigma$ (red) and $3\sigma$ (cyan). Structural changes consistent with isomorphous difference Fourier maps are represented by black arrows, where larger arrowheads represent the larger structural changes. (D to F) Interatomic distances (angstroms) of the OECs in the (D) S$_1$, (E) S$_2$, and (F) S$_3$ states. Blue and red lines indicate elongation and shortening of the interatomic distances compared with the structure in the precedent S$_s$ state. Presumed Mn (+4) and Mn (+3) cations are shown. (G) The $F_{\text{obs}} - F_{\text{calc}}$ difference Fourier maps contoured at $-2.2\sigma$ (red) and $+2.2\sigma$ (cyan) after structural refinement by fixing the distance between O5 and O6 at 1.3 Å (superoxide), 1.5 Å (peroxo), 1.7 Å, 1.9 Å (oxyl/oxo), and 2.4 Å (hydroxo/oxo), respectively. Values in parentheses are temperature factors (square angstroms) of O5 and O6. Black arrows indicate the residual electron densities that were affected by the interatomic distances between O5 and O6. (H) The $F_{\text{obs}} - F_{\text{calc}}$ difference Fourier maps contoured at $5\sigma$ (blue) when both O5 and O6, or just O6, were omitted. Colors used here are the same in all figures unless otherwise noted.

**Structural determination**

Single-shot diffraction images were collected in a fixed-target data collection manner at a cryogenic temperature ($5\degree$K), in which PSII microcrystals were used (fig. S1, see supplementary materials and methods). Compared with SFX of PSII using a grease matrix as the injection medium at room temperature ($20\degree$), this method reduced the sample consumption by one order of magnitude and ensured low background images, allowing us to collect diffraction images with high hit and index rates, which yielded 2.15-Å resolution datasets for PSII in the S$_1$, S$_2$, and S$_3$ states (dataset 1 in tables S1 and S2). We collected datasets of a 3F...
state and S₁, S₂, and S₃ states (dataset 2) at 2.35- to 2.50-Å resolution independently to confirm the reproducibility of the light-induced structural changes and to examine the structural changes beyond the S₃ state.

Strong peaks in the isomorphous difference Fourier maps calculated between each state (Fig. 2, A to C, and fig. S3) indicate successful detection of the structural changes induced by the flash illuminations. Most peaks were localized in the vicinity of OEC, in agreement with the previous observations at room temperature by the SFX method (11) (Figs. 2 to 4 and fig. S3). However, structural changes in the quinone Qₐ-binding site were diminished substantially, reflecting the relaxed protein environment due to the longer delay time after flash illumination in the present study (1 s in the fixed-target method versus 10 ms in the SFX method). Pairs of positive and negative density peaks were more clearly visible in the current difference maps around the OEC owing to the high resolution achieved as well as the high isomorphism between the different S-state datasets. Changes in the interatomic distances between the four Mn atoms accompanying the Sᵢ-state transition (table S4) reflect changes of the oxidation states of the Mn ions. Difference maps between sequential states allowed us to define the order of serial structural changes induced by S-state progression.

### The S₁ and S₂ states

The Mn-Mn distances in the S₁ state in our structure (Fig. 2D and table S4) are similar to previous results (5, 11, 16, 20), with the exception being that the shortest distance (Mn1-Mn2, 2.60 Å) is slightly shorter than what was previously reported. The S₂ state may adopt either an open or a closed cubane form according to theoretical studies (21, 22). The open cubane corresponds to the structure giving rise to the S = 1/₂, B = 0 electron paramagnetic resonance (EPR) multiline signal (S, spin; g, g-factor, a dimensionless quantity that characterizes the magnetic moment and angular momentum of an atom), and the closed cubane to the S = 1/₂, B = 4.1 signal (21, 22). The S₂ state in our structure is in the open cubane form, in agreement with the absence of the B = 4.1 EPR signal in cyano-bacterial PSI1 under normal conditions (23). Difference density analysis reveals that, upon S₁-to-S₂ transition, Mn₄ is shifted toward Glu³³³ and Ca is moved toward Yz (Tyr¹⁶¹) slightly (Fig. 2A). There is a shift of O⁵ toward Mn₄ and a weakening or breakage of the oxy bridge between O⁵ and Mn¹ in the S₂ state (Fig. S4). These changes are consistent with Mn₄ oxidation during the S₁-to-S₂ transition, giving rise to a bipyramidal five coordinated Mn¹ and an open cubane OEC structure consistent with a charge distribution of (Mn¹, Mn², Mn³, Mn⁴) = (III, IV, IV, IV). Slight changes in Mn-Mn distances are observed in the S₂ state (Fig. 2E), resulting in an increased homogeneity in these distances consistent with x-ray absorption fine structure measurements (24).

In addition to the structural changes of OEC, strong negative densities were found for two water molecules, W₆₆₅ and W₅₇₁, that are hydrogen-bonded with ligand residues of the OEC (Fig. 2, A and C). These changes have been found in the S₁-to-S₃ transition (11), but the present results show that these two water molecules already become highly disordered in the S₁-to-S₃ transition; therefore, the possibility of W₆₆₅ as the substrate for O₂ oxidation is unlikely. W₆₆₅ is the second water molecule from O₄ within the 15-Å-long O₄ water chain that ends at a water cluster consisting of five water molecules exposed to the luminal solution (Fig. 1). This water cluster may accept a proton released from OEC as a protonated Eigen cation through the Grotthuss-type proton transfer (25), consistent with Fourier transform infrared spectroscopy, which showed changes in a highly polarized hydrogen-bond network (26–28) or possible formation of a nH₂O(H₂O)⁺ cluster in the S₂ state (29, 30). Despite a long distance between the water cluster and the OEC, change was observed in both electron density and the shape of the water cluster (Fig. 3, A and B). These changes may result from positive charge accumulated during the S₁-to-S₃ transition, possibly by the ejection of a proton from the OEC through W₆₆₅. The increased mobility of W₆₆₅ may be required

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**Fig. 3. Structural changes in the O₄ channel.** (A) Fₐₕₒₛ – Fₜₒₛ isomorphous difference Fourier map of 1F minus dark contoured at 3σ superimposed with the hydrogen-bonding network in the O₄ channel. Dislodged water molecules are highlighted by blue circles. (B) Fₚₒₛ – Fₗₒₛ when water or O₆ were omitted (top) or Fₚₒₛ – Fₜₒₛ (bottom) difference density values at water sites in the O₄ channel. rmsd, root mean square deviation. (C) Possible proton transfer mechanism in the O₄ channel. Hydrogen-bonding networks before (left) and after (right) proton transfer. The putative proton stored at the water cluster is depicted in green and indicated by a red arrow.
to prevent the backflow of the proton by disconnecting the water chain (Fig. 3C) and is accompanied by a slight shift of the side chain of CP43 Glu354 hydrogen-bonded with W665. W571 also increases its mobility on the S2-to-S3 transition (Figs. 2, A and B, and fig. S3, B and C). Mn1, Mn2, and Mn3 are static, but Mn4 is moved toward Ser359 (Fig. 2B), resulting in an increase in the Mn1-Mn4 distance (Fig. 2, E and F). Flipping of the side chain of Glu189, also seen at room temperature (II), provides an open space in the vicinity of O5, enabling the insertion of O6 (Fig. 4, A and B). The improved resolution of the dataset allowed us to identify the position of O6 clearly in both Fobs - Fcalc and Fobs - Fcalc difference maps and thus determine its chemical structure unambiguously (Fig. 2, B, C, and H). By altering the O5-O6 distance and examining the residual densities in the Fobs - Fcalc difference Fourier map, we found that a distance of 1.9 Å resulted in the weakest residual densities (Fig. 2G) (see materials and methods). This distance is slightly longer than the 1.5 Å we reported previously (II) and consistent with an oxyl/oxo pair for the O5 and O6 species. Distances for superoxo (1.5 Å), peroxy (1.5 Å), and hydroxy/oxo (2.4 Å) species can be excluded on the basis of their increased residual densities. Furthermore, a hydroxy/oxo pair cannot be accommodated by the current structure owing to the limited space available, unless the interatomic distance between O5 and Mn4 is shorter by 0.3 Å. A peroxo species fits with the electron density similarly to that of the oxyl/oxo species, explaining why we could not discriminate in the previous study (II); however, the oxyl/oxo species resulted in less residual electron densities in the Fobs - Fcalc map and evenly distributed temperature factors for O5 and O6. The interatomic distances of OEC in the S3 state agree well with those of the theoretically optimized structure of S3 when the oxyl/oxo species was assumed in the open cubane form (table S5) (31). Thus, we conclude that the O5 and O6 pair is in an oxyl/oxo form, which would be consistent with an oxyl/oxo coupling mechanism for the O=O bond formation (31).

Movement of D1 Glu189, the only monodentate carboxylate ligand of the Mn4CaO5 cluster, by 0.5 Å during the S2-to-S3 transition

**The oxyl/oxo species in the S3 state**

Larger difference densities were observed around the OEC for the S2-to-S3 transition than for the S1-to-S2 transition (Fig. 2, A and B, and fig. S3, B and C). Mn1, Mn2, and Mn3 are static, but Mn4 is moved toward Ser359 (Fig. 2B), resulting in an increase in the Mn1-Mn4 distance (Fig. 2, E and F). Flipping of the side chain of Glu189, also seen at room temperature (II), provides an open space in the vicinity of O5, enabling the insertion of O6 (Fig. 4, A and B). The improved resolution of the dataset allowed us to identify the position of O6 clearly in both Fobs - Fcalc and Fobs - Fcalc difference maps and thus determine its chemical structure unambiguously (Fig. 2, B, C, and H). By altering the O5-O6 distance and examining the residual densities in the Fobs - Fcalc difference Fourier map, we found that a distance of 1.9 Å resulted in the weakest residual densities (Fig. 2G) (see materials and methods). This distance is slightly longer than the 1.5 Å we reported previously (II) and consistent with an oxyl/oxo pair for the O5 and O6 species. Distances for superoxo (1.5 Å), peroxy (1.5 Å), and hydroxy/oxo (2.4 Å) species can be excluded on the basis of their increased residual densities. Furthermore, a hydroxy/oxo pair cannot be accommodated by the current structure owing to the limited space available, unless the interatomic distance between O5 and Mn4 is shorter by 0.3 Å. A peroxo species fits with the electron density similarly to that of the oxyl/oxo species, explaining why we could not discriminate in the previous study (II); however, the oxyl/oxo species resulted in less residual electron densities in the Fobs - Fcalc map and evenly distributed temperature factors for O5 and O6. The interatomic distances of OEC in the S3 state agree well with those of the theoretically optimized structure of S3 when the oxyl/oxo species was assumed in the open cubane form (table S5) (31). Thus, we conclude that the O5 and O6 pair is in an oxyl/oxo form, which would be consistent with an oxyl/oxo coupling mechanism for the O=O bond formation (31).

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![Fig. 4. Structural changes in the water-inlet O1 channel and around the Ca2+ ion. (A) Isomorphous difference map (3σ) superimposed with the O1 channel. O6 and two water molecules that appear in the S3 state are marked with a red dashed circle. (B) Model of structural changes in the O1 channel. Weak interactions between Glu189 and Ca2+, and between Glu189 and O6, are shown as dashed lines. Possible hydrogen atom transferred from O6 to Glu189 is shown as a solid green line. (D) Schematic structures of OEC and the states of the O1 and O4 channels](http://science.sciencemag.org/).
causes an elongation of the distance between its O1E and Ca$^{2+}$ from 3.1 Å in the S1 state to 3.5 Å in the S3 state (Fig. 4C). The Ca$^{2+}$ ion can be considered to have a coordination number of 7.5 or pseudo-eighth-coordinated in the S1 state, because it has seven "normal" ligands plus D1 Glu$^{189}$. In the S3 state, the interaction between D1 Glu$^{189}$ and Ca$^{2+}$ is much weaker, but the newly inserted O6 provides an eighth ligand to Ca$^{2+}$ with a distance of 2.6 Å (Fig. 4C), which is made possible by the ability of Ca$^{2+}$ to adopt coordination numbers from four to eight (32). The two hydrogen atoms brought by O6 are, respectively, ejected from the active site during the S2-to-S3 transition and accepted by D1 Glu$^{189}$, which forms a hydrogen bond with O6 (Fig. 4C), giving rise to the oxyl/oxo species between O6 and O5.

The mechanism for substrate water delivery

Flipping of the Glu$^{189}$ side chain is correlated with motions in a short loop of CP43, including CP43 Val$^{320}$, that restrict the size of the O1 channel (Fig. 4, A and B). Paired positive and negative densities were found around CP43 Val$^{320}$ indicating a movement of this residue toward Glu$^{189}$ by 0.5 Å in the S3 state. This movement substantially widens the channel radius (Fig. 4, A and B), which may allow water molecules to come in during the S3 state transition. Strong negative difference densities were found in positions overlapping with W503, W547, and W554 as well as glycerol-3 phosphate (Gol526) present in the channel, reflecting increased mobility of these molecules in response to the channel opening in the S3 state. Two positive difference densities in the hydrogen-bonded water network indicate the insertion of water molecules W666 and W667 into the O1 channel (Fig. 4, A and B). The O1 channel likely serves as a conduit for substrate water entry into the OEC, and the residues around CP43 Val$^{320}$ may serve as a "valve" to control the water-inlet channel. Movements in this region coincide with incorporation of O6 into the open cubane structure of OEC during the S2-to-S3 transition. Thus, Glu$^{189}$ plays a pivotal role in coupling oxidation of the OEC with the opening of the water channel and delivery of the substrate into the OEC.

Outlook

The structural changes related to proton release, water inlet, and O=O bond formation during the S-state cycle are summarized in Fig. 4D. The displacement of W665 in the O4 channel suggests a proton transfer from the OEC upon S1-to-S2 transition. Considering that theoretical calculations favor O5 as a hydroxide ion in S1 and an oxide ion in the open cubane S2 structure (33), and that there is no proton release to the bulk solution in the S2-to-S3 transition, a proton is likely ejected from the O5 site and stored as a protonated Eigen cation in PSIIL. The structural changes observed in the O4 channel thus provide insights into the timing of proton release and an elegant way to prevent the backflow of the released proton. Insertion of O6 occurs in the S2-to-S3 transition, providing an oxyl/oxo species in the S3 state. Structural changes of the Glu$^{189}$ side chain serve to couple oxidation of the catalytic site with substrate water access, proton release, and O=O bond formation via the oxyl/oxo coupling mechanism. The structure and O=O bond formation mechanism revealed here should serve as an important blueprint for rational design of artificial catalysts that have the capability for oxidizing water by visible light.

REFERENCES AND NOTES


ACKNOWLEDGMENTS

The x-ray free electron laser experiments were performed at SACLA with the approval of the Japan Synchrotron Radiation Institute (JASRI) (proposal nos. 2016A051, 2016B070, 2017A0400, 2017B0015, 2017B0028, 2018A009, and 2018A010), and we thank the staff at SACLA for their help. The best condition for data collection was determined at beamlines 41XU and 44XU in Spring-8 (proposal nos. 2016A0254, 2016A0662, 2016B0254, 2016B0621, 2017A235, 2017A6724, 2017B6724, 2018A2530, 2018B0820, 2018B2530, and 2018B6822). Funding: This research was supported by the Platform Project for Supporting Drug Discovery and Life Science Research [Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)] from AMED under grant JP18am0101070. This research was also supported by KAKENHI grants JP16H06162, JP16H06206, and JP17H05884 (M.S.); JP17H03434 (J.-R.S.); and JP16K21181 (F.A.). Support was also provided by JST, PREST grants JPMJPR18G8 (M.S.) and JPMJPR16P1 (F.A.), and RIKEN Pioneering Project "Dynamic Structural Biology" to M.K. and M.Yam..

Author contributions: J.-R.S., M.S., M.Yam., and H.A. conceived the project. G.U., H.M., S.B., T.Ku., H.A., and M.Yam. developed the data collection setup. M.K., T.N., and T.N. set up a laser system for the sample preparation. K.T. and M.Yab. prepared the samples and grew the crystals. M.S., H.L., Y.Y., S.I., L.-J.Y., and J.-R.S. collected the diffraction data. K.Yamas., H.L., T.Y., and M.S. processed the diffraction data. M.S. analyzed the structure. H.I. and K.Yamaguchi, the authors declare no competing interests. Data and materials availability: The atomic coordinates and structure factors have been deposited in the Protein Data Bank under the following IDs: 6JLJ for S1 (dataset 1), 6JLX for S2 (dataset 2), 6JLL for S3 (dataset 1), 6LIM for S2 (dataset 2), 6LJQ for S1 (dataset 2), and 6LPF for 3F. All other data used in this study are presented in the main text or the supplementary materials.

SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/366/6463/334/suppl/DC1

Funding: This research was supported by the Platform Project for Supporting Drug Discovery and Life Science Research [Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)] from AMED under grant JP18am0101070. This research was also supported by KAKENHI grants JP16H06162, JP16H05206, and JP17H05884 (M.S.); JP17H03434 (J.-R.S.); and JP16K21181 (F.A.). Support was also provided by JST, PREST grants JPMJPR18G8 (M.S.) and JPMJPR16P1 (F.A.), and RIKEN Pioneering Project “Dynamic Structural Biology” to M.K. and M.Yam. The authors declare no competing interests. Data and materials availability: The atomic coordinates and structure factors have been deposited in the Protein Data Bank under the following IDs: 6JLJ for S1 (dataset 1), 6JLX for S2 (dataset 1), 6JLL for S3 (dataset 1), 6LIM for S2 (dataset 2), 6LJQ for S1 (dataset 2), and 6LPF for 3F. All other data used in this study are presented in the main text or the supplementary materials.

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15 April 2019; accepted 9 September 2019
10.1126/science.aae6998
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DOI: 10.1126/science.aax6998

**Inspecting S states in photosynthesis**

Oxygenic photosynthesis uses a \( \text{Mn}_4\text{CaO}_5 \) cluster in the oxygen-evolving complex to extract electrons from water and produce dioxygen. Visualizing each of the chemical states in this process, \( S_0 \) to \( S_4 \), and assigning chemical identities and mechanisms on the basis of structures has been a challenge addressed recently by work at x-ray free-electron lasers. Suga *et al.* used serial crystallography at cryogenic temperatures to trap and determine the structures of several stable states during photosystem II water oxidation (see the Perspective by Britt and Marchiori). Changes around the water cluster already happen in the \( S_3 \) state and set the stage for water insertion that occurs during transition to the \( S_3 \) state. A short 1.9-angstrom distance between the two oxygen atoms in the \( S_3 \) state is consistent with theoretical studies supporting an oxyl/oxo mechanism for oxygen-oxygen coupling.

"Science, this issue p. 334; see also p. 305"

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