



# Untangling the sequence of events during the $S_2 \rightarrow S_3$ transition in photosystem II and implications for the water oxidation mechanism

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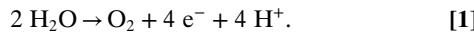
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**In oxygenic photosynthesis, light-driven oxidation of water to molecular oxygen is carried out by the oxygen-evolving complex (OEC) in photosystem II (PS II). Recently, we reported the room-temperature structures of PS II in the four (semi)stable  $S$ -states,  $S_1$ ,  $S_2$ ,  $S_3$ , and  $S_0$ , showing that a water molecule is inserted during the  $S_2 \rightarrow S_3$  transition, as a new bridging O(H)-ligand between Mn1 and Ca. To understand the sequence of events leading to the formation of this last stable intermediate state before  $O_2$  formation, we recorded diffraction and Mn X-ray emission spectroscopy (XES) data at several time points during the  $S_2 \rightarrow S_3$  transition. At the electron acceptor site, changes due to the two-electron redox chemistry at the quinones,  $Q_A$  and  $Q_B$ , are observed. At the donor site, tyrosine  $Y_2$  and His190 H-bonded to it move by 50  $\mu$ s after the second flash, and Glu189 moves away from Ca. This is followed by Mn1 and Mn4 moving apart, and the insertion of  $O_X(H)$  at the open coordination site of Mn1. This water, possibly a ligand of Ca, could be supplied via a “water wheel”-like arrangement of five waters next to the OEC that is connected by a large channel to the bulk solvent. XES spectra show that Mn oxidation ( $\tau$  of ~350  $\mu$ s) during the  $S_2 \rightarrow S_3$  transition mirrors the appearance of  $O_X$  electron density. This indicates that the oxidation state change and the insertion of water as a bridging atom between Mn1 and Ca are highly correlated.**

photosynthesis | photosystem II | water oxidation | oxygen-evolving complex | X-ray free electron laser

**D**ioxygen, which supports all aerobic life, is abundant in the atmosphere because of its constant regeneration via photosynthetic water oxidation in plants, algae, and cyanobacteria. The water-splitting chemistry occurs in the oxygen-evolving complex (OEC) of photosystem II (PS II; Fig. 1*A*), which contains a heteronuclear, oxo-bridged  $Mn_4Ca$  cluster that catalyzes the reaction:



To allow this reaction to take place, the OEC accumulates four oxidizing equivalents, stepping through intermediates that are referred to as the  $S$ -states ( $S_i$ ,  $i = 0$  through 4) (Fig. 1*B*) (1, 2). These oxidation reactions are driven by the light-induced charge separations in the reaction center of PS II that comprises the

chlorophyll  $a$  moiety  $P_{680}$  (most likely consisting of the four excitonically coupled chlorophylls  $P_{D1}$ ,  $P_{D2}$ ,  $Chl_{D1}$ , and  $Chl_{D2}$ ) and the neighboring pheophytin molecules  $Pheo_{D1/D2}$  as well as the two plastoquinones  $Q_A$  and  $Q_B$ . The one-electron photochemistry at

## Significance

**A new bridging oxygen ligand is incorporated between one of the Mn atoms and Ca in the  $Mn_4Ca$  cluster during the transition from the one-photon induced  $S_2$  intermediate state to the two-photon induced  $S_3$  state in the catalytic water oxidation reaction in photosystem II. However, the sequence of events leading to this change is not known. Here we report an X-ray crystallography and spectroscopy study at room temperature using an X-ray free electron laser to collect a “molecular movie” of the structural and oxidation state change steps leading to the insertion of this new oxygen bridge, in the 50  $\mu$ s to 200 ms time scales after photon absorption, which triggers the  $S_2 \rightarrow S_3$  state transition.**

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The authors declare no competing interest.

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Data deposition: X-ray diffraction datasets and associated models have been deposited in the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (<https://www.rcsb.org>) under PDB ID codes **6W1O** for the 0F, **6W1P** for the 1F, **6W1Q** for the 2F(50  $\mu$ s), **6W1R** for the 2F(150  $\mu$ s), **6W1T** for the 2F(250  $\mu$ s), **6W1U** for the 2F(400  $\mu$ s), and **6W1V** for the 2F(200 ms) data.

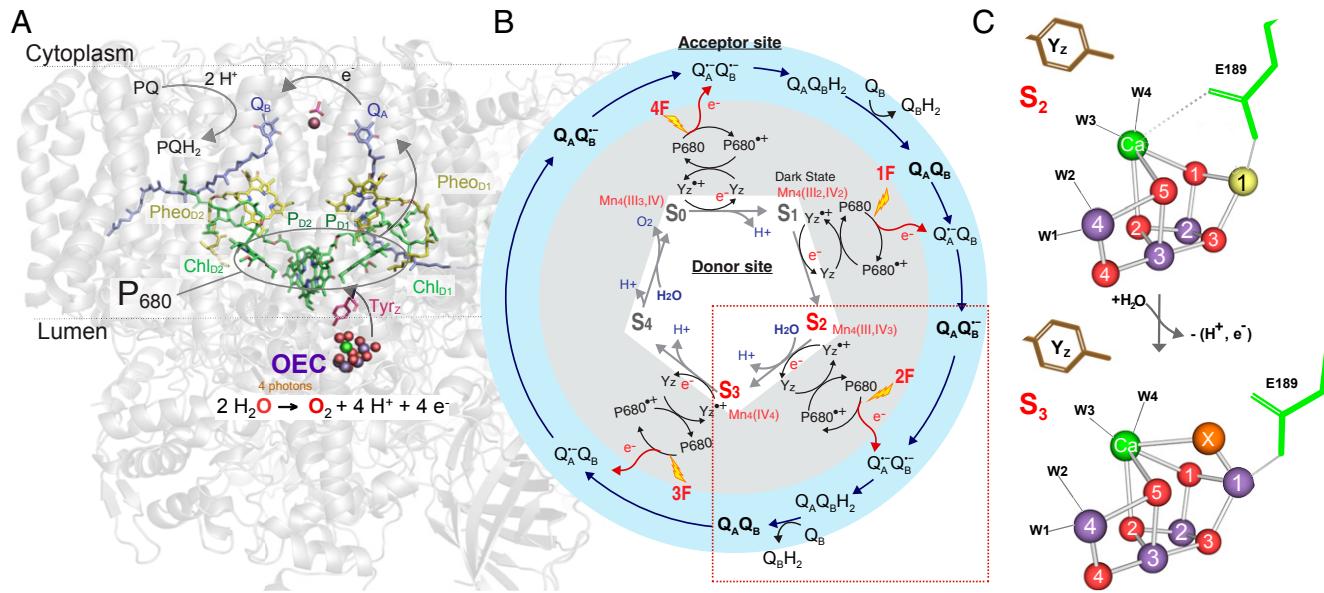
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**Fig. 1.** The S<sub>2</sub> → S<sub>3</sub> transition in PS II. (A) The structure of PS II with the membrane-embedded helices and the large membrane extrinsic regions on the luminal side of PS II are shown as cartoon in gray and in color the main electron transfer components involved in charge transfer (P<sub>680</sub>, Pheo) and stabilization with the acceptor quinones, Q<sub>A</sub> and Q<sub>B</sub>, toward the cytoplasm, and Y<sub>Z</sub> and OEC with catalytic Mn<sub>4</sub>Ca cluster on the donor side at the interface between the membrane and the lumen-bound part of PS II. (B) Kok cycle of the water oxidation reaction that is triggered by the absorption of photons (nanosecond light flashes, 1F to 4F) in the antenna and the reaction center P<sub>680</sub>. The white and gray areas show the redox changes of the OEC (S<sub>1</sub> to S<sub>0</sub>) and Y<sub>Z</sub> together with the charge separation reactions, while the outer blue circle shows the concomitant acceptor side chemistry of the quinones. The S<sub>2</sub> → S<sub>3</sub> transition, marked by the red box, is discussed in detail in the main text. (C) The structures of the Mn<sub>4</sub>Ca cluster, showing the major changes between the S<sub>2</sub> and S<sub>3</sub> states (Mn<sup>3+</sup> yellow, Mn<sup>4+</sup> purple, Ca<sup>2+</sup> green, O red; W1, 2, 3, 4 are water ligands to Mn4 and Ca). The Glu189 residue of the D1 protein as a ligand of Ca moves away during the transition with the insertion of oxygen (O<sub>X</sub> = O<sup>2-</sup> or OH<sup>-</sup>) as a new bridging ligand between Mn1 and Ca (adapted from ref. 20).

the reaction center is coupled to the four-electron redox chemistry at the OEC via a redox active tyrosine residue D1-Tyr161, also known as Y<sub>Z</sub>. Once four oxidizing equivalents are accumulated (S<sub>4</sub>-state), the formation of dioxygen occurs spontaneously and leads to the release of O<sub>2</sub> and the reformation of the S<sub>0</sub>-state. At the electron acceptor side of PS II, the electrons created in the charge separation process are transferred to plastoquinone Q<sub>A</sub> and subsequently to plastoquinone Q<sub>B</sub>. After two light-induced charge separations, the dihydroplastoquinol, Q<sub>B</sub>H<sub>2</sub>, formed at the Q<sub>B</sub> site is replaced by a new plastoquinone molecule (Fig. 1 A and B) (3, 4).

The significance of the S<sub>2</sub> → S<sub>3</sub> transition among the four catalytic steps of the Mn<sub>4</sub>Ca cluster, is the fact that a water, possibly a substrate, is delivered via a water channel and incorporated into the OEC (Fig. 1 A and B). Biophysical experiments over the past decades have shown that small perturbations of the OEC can block or severely slow down the rate of this transition, likely by altering the protonation state of the H-bonding network around the OEC. This is caused, for example, by Cl<sup>-</sup> depletion (5, 6), substitution of Ca<sup>2+</sup> by Sr<sup>2+</sup> in the OEC (7, 8), low temperature (9), and a number of site-directed mutations (10). In addition, there is substantial evidence that the S<sub>2</sub> → S<sub>3</sub> transition is, by far, the least facile step in the catalytic cycle as it is coupled to the largest reorganization energy, probably due to the water insertion event (11–14).

Since the advent of X-ray free electron lasers (XFELs) there have been efforts to study intermediate states of PS II (15–21). Recently, we reported the crystal structures of all stable intermediates of PS II during the Kok cycle that appear under physiological temperature and two transient time points during the S<sub>2</sub> → S<sub>3</sub> transition (20). These studies have started unveiling the stepping-stone structures of the catalytic process. The binding of one additional water to the Mn<sub>4</sub>Ca cluster was reported in the S<sub>3</sub> state on the basis of XFEL experiments by both Shen and coworkers and by us

(19–21). This binding has been predicted by biophysical and computational studies (14, 22–31). However, the chemical form we observed for this newly bound water-derived species differs from the predictions and the initial experimental results; unlike the predicted terminal hydroxo to Mn1 (23, 32) or the experimentally proposed complexed peroxy/superoxy moiety formed together with the O<sub>5</sub> bridge (19), we found that the insertion of the new water leads to the formation of an oxo or hydroxo bridge between Mn1 and Ca (Fig. 1C) (20). This unexpected binding motif for O<sub>X</sub>/O<sub>6</sub> was recently confirmed by Suga et al. (21). Our study also revealed several structural changes that accompany this water insertion event, including the changes in the ligation environment of Ca and metal-metal distances within the Mn<sub>4</sub>Ca cluster (20).

However, the exact sequence of changes in the complex S<sub>2</sub> → S<sub>3</sub> structural rearrangement and the cause for each of the individual steps remains unresolved. Recent time-resolved photothermal beam deflection and Fourier transform infrared (FTIR) experiments have shown that the oxidation of Y<sub>Z</sub> by P<sub>680</sub><sup>•+</sup> is followed by proton transfer/release in the S<sub>2</sub> → S<sub>3</sub> transition, and that only thereafter can the electron transfer from Mn to Y<sub>Z</sub><sup>•</sup> occur (33, 34). The order of the two other important steps of this transition, oxidation of Mn and insertion of water, is not clear yet, and proposals for either oxidation first or insertion first have been put forward (26, 30, 35–39). Additionally, it was suggested that the cluster undergoes some rearrangement between a “right-open” or “open-cubane” and a “left-open” or “closed-cubane” structure during the S<sub>2</sub> → S<sub>3</sub> transition (*SI Appendix*, Fig. S1) (40).

The observation from our earlier structural study (20) led us to realize that untangling the sequence of events in the S<sub>2</sub> → S<sub>3</sub> transition is critical for understanding why one state proceeds to another following a certain pathway, what structural and/or electronic features influence the reaction kinetics, and eventually how is the process fine-tuned. Combining XFEL-based room-temperature serial femtosecond crystallography (SFX) with X-ray

emission spectroscopy (XES) provides a powerful tool to capture and untangle key intermediate structures during this process. Utilizing these methods we report here high-resolution structures and oxidation states of PS II at four time points (50, 150, 250, and 400  $\mu$ s) during the  $S_2 \rightarrow S_3$  transition, probing the kinetics of the electronic and structural changes.

## Results and Discussion

**Structural Changes during the  $S_2 \rightarrow S_3$  Transition.** We collected seven different SFX datasets with resolutions ranging from 2.01 to 2.27 Å (*SI Appendix*, Table S1 and Figs. S2–S4). These contained in addition to the dark-adapted ( $S_1$ ), single-illuminated ( $S_2$ ), and doubly illuminated ( $S_3$ ) state (collected 200 ms after the first and second pump laser flash, respectively) four transient state datasets at time points (50, 150, 250, and 400  $\mu$ s after the second flash) during the  $S_2 \rightarrow S_3$  transition. For the analysis of these datasets, we incorporated the ensemble refinement method described by Brewster et al. (ref. 41; also see *Methods*) that takes care of the dynamics of detector metrology and the beam parameters that are fundamental to the XFEL experiment. The structural changes at the donor side, previously observed at 150 and 400  $\mu$ s at resolutions of 2.20 and 2.50 Å, were reproduced in the current datasets. At the same time, the sequence of structural changes is more evident due to the additional time points (50  $\mu$ s and 250  $\mu$ s) and the improved resolution.

**$Q_A/Q_B$  Acceptor Quinone Sites.** Although the plastoquinones  $Q_A$  and  $Q_B$  have identical chemical structures, their binding constants inside PS II are quite different, due to their significantly different binding pockets. The binding energies of  $Q_A$  and  $Q_B$  have been calculated to be  $-56.1$  kcal/mol and  $-37.9$  kcal/mol, respectively (42). The lower binding affinity of  $Q_B$  is reflected in our data by the less-well-defined 2Fo-Fc maps and the average B-factor for the quinone head group being about twice as high for  $Q_B$  compared to  $Q_A$ .

The second flash leads to the reduction of  $Q_A$  to  $Q_A^-$  and subsequently to the  $Q_A^-$  to  $Q_B^-$  electron transfer, protonation of  $Q_B^{2-}$ , and the exchange of  $Q_BH_2$  with  $Q_B$ .  $Q_A^-$  formation is clearly seen at 50  $\mu$ s by the twist of  $Q_A^-$  relative to the  $Q_A$  position and the  $\sim 0.2$  Å decrease of the H-bonding distances to D2-His214 and the backbone nitrogen of D2-Phe261 that is expected due to the increased electronegativity of the semiquinone groups. It is interesting that the isomorphous difference maps around  $Q_A/Q_A^-$  change much less than observed for  $Q_B$  upon  $Q_B^-$  formation (Fig. 2). This suggests that the more rigid protein pocket around  $Q_A$  (showing a consistently smaller B-factor compared to the  $Q_B$  binding pocket) does not adjust significantly to the reduction of the quinone, which in turn could contribute to the increased reduction potential of  $Q_A^-$  compared to that of  $Q_B^-$ .

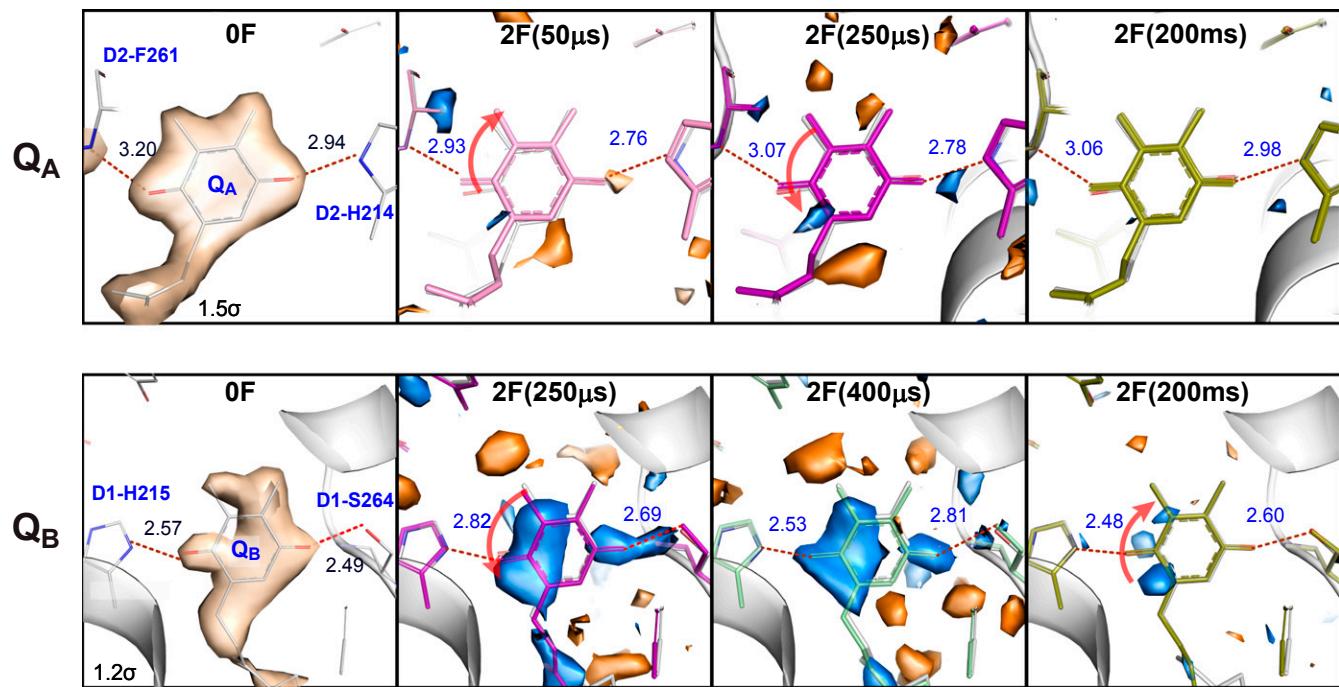
Since the  $Q_A^-$  to  $Q_B$  and  $Q_A^-$  to  $Q_B^-$  electron transfers were reported to take place in PS II with time constants of 0.3 to 9 ms (43, 44), the changes observed between 50  $\mu$ s and 400  $\mu$ s (Fig. 2 and *SI Appendix*, Fig. S5) reflect the rearrangements of protein–H-bonding due to the reduction of  $Q_A$  and the onset of the electron transfer between  $Q_A^-$  and  $Q_B^-$ . The negligible differences observed in the 2F-0F isomorphous difference maps at the 200 ms time point at the  $Q_B$ -site show that the electron transfer from  $Q_A^-$  to  $Q_B^-$  and the exchange of  $Q_BH_2$  vs a new oxidized quinone of the quinone pool present in the crystal are complete at this stage (exchange with an oxidized quinone is possible due to the presence of a small plastoquinone pool inside the crystal preparations, as shown previously in refs. 18, 20, and 45).

**Tyrosine  $Y_Z$  (D1-Tyr161)/His190 Site.** At the donor site the tyrosine residue,  $Y_Z$  (D1-Tyr161), which is located between  $P_{680}$  and the OEC (Fig. 1A), plays a critical role for electron transfer between these two sites (46–48). Fig. 3 shows the omit maps and models

of the  $Y_Z$ , D1-His190, and D1-Asn298 residues, overlaid with the 0F( $S_1$ ) omit map and structural model. Oxidation of  $Y_Z$  after the charge separation at  $P_{680}$  has a half-life in the nanosecond to microsecond range (49–51). Therefore, small, but noticeable, changes in this area at 2F(50  $\mu$ s) correspond to the changes induced by the oxidation of  $Y_Z$  after electron transfer to  $P_{680}$  which is coupled to the transfer of the phenolic proton to the H-bonding partner His190 (see *SI Appendix*, Fig. S6 for a more detailed view of the H-bonding environment of  $Y_Z$ ). A tilt of the phenol ring of  $Y_Z$  by about 3.5° is observed in comparison to the 0F ( $S_1$ ) data (Fig. 3). Overall, the largest changes in the electron density are observed at 150  $\mu$ s after the second flash [2F(150  $\mu$ s)], implying a significant movement at the His190 and Asn298 side chains. Accordingly, the structural model for the 2F(150  $\mu$ s) data shows a tilt/shift of the imidazole ring of His190 by  $\sim 0.5$  Å in comparison to the  $S_1$  structure and a shift in the side chain of Asn298 of  $\sim 0.3$  Å. These changes may be related to subsequent additional protonation state changes in the region of  $Y_Z$ -His190-Asn298 that are related to the oxidation of the  $Mn_4Ca$  cluster and the insertion of a new water into the  $O_x$  position (*Possible Routes for  $O_x$  Insertion*). This path has been proposed as a proton transfer pathway by Umena et al. (52). The strong hydrogen bond between the phenolic O-group of  $Y_Z$  and the  $N\tau$  of His190 makes proton-coupled electron transfer possible upon charge separation (29). In the 250  $\mu$ s and 400  $\mu$ s data, D1-His190 and D1-Asn298 have returned to their original positions and a slow relaxation of  $Y_Z$  back toward the conformation before charge separation can be observed that reflects the reduction of  $Y_Z^{Ox}$  by the  $Mn_4Ca$  cluster. This process is complete in the fully evolved 2F ( $S_3$ ) state. The observed changes involving Asn298 in a hydrogen-bond network that is part of a proton transfer pathway away from  $Y_Z$  are also in line with mutant studies showing a partial inhibition of the  $S_2 \rightarrow S_3$  and  $S_3 \rightarrow S_0$  transitions (that both involve proton release) in an Asn298Ala mutant (53). The observed time scale of  $\sim 50$  to 200  $\mu$ s for structural changes around His190 and Asn298 is well in line with proposed time scales for proton transfer events in the  $S_2 \rightarrow S_3$  transition (29, 34). It is also worth noting that the changes at His190 are not restricted to the imidazole side chain. They are also connected to a shift of the protein backbone by  $\sim 0.4$  Å. This shift in turn is directly connected to a shift of the backbone of the neighboring Glu189 that shows significant side-chain movement in the  $S_2 \rightarrow S_3$  transition (discussed below).

**OEC.** Fig. 4 shows the refined structure around the OEC at each time point, overlaid with isomorphous difference maps relative to the 0F data. Selected atomic distances are shown in Fig. 5A. At 50  $\mu$ s after the second flash, the first observable change in this area involves the Glu189 residue that is 3.8 Å away from the phenolic group of  $Y_Z$ . One carboxylate oxygen of this residue ligates to Mn1, while the second carboxylate oxygen has a weak interaction with Ca in the  $S_1$ (0F) and  $S_2$ (1F) state (2.8 Å), and with W25 (2.6 Å). After the second flash, the Glu189 side chain moves away from Ca but remains as a monodentate ligand to Mn1. The distance to Ca changes from  $\sim 2.8$  (1F,  $S_2$ ) to  $\sim 3.2$  Å [2F(50  $\mu$ s)] (Fig. 5A). It shows that the position of the Glu189 carboxylate oxygen facing Ca is highly flexible, and its electrostatic interaction with Ca becomes even weaker during the  $S_2 \rightarrow S_3$  transition. We observe that 150  $\mu$ s after the second flash, Glu189 also moves away from W25 by  $\sim 0.3$  Å. This is compatible with the time scale of  $Y_Z$  oxidation whereas the subsequent transfer of the positive charge to the OEC did not start yet. It should be noted that changes in side-chain orientation are connected with backbone position changes in both Glu189 and His190 and as they are direct neighbors possibly influence each other (discussed above).

The phenolic oxygen of  $Y_Z$  has three neighboring contacts that can form hydrogen bonding interactions: His190, W25, and W4 (*SI Appendix*, Fig. S6). The change in the Glu189–W25 distance suggests that W25 is acting as a proton relay for the subsequent  $Y_Z$  protonation step and may subsequently facilitate W3 deprotonation.



**Fig. 2.** The acceptor side of PS II. 2Fo-Fc electron density map (contoured at  $1.5\sigma$  or  $1.2\sigma$  due to different mobility of  $Q_A$  and  $Q_B$ ) of the OF data, Fo-isomorphous difference maps (contoured at  $+3\sigma$  [blue] and  $-3\sigma$  [orange]) of 2F(time point)-OF diffraction data and refined models for OF and 2F(time point) data in the region of  $Q_A$  (Top) and  $Q_B$  (Bottom) on the acceptor side of PS II. Red arrows indicate movement of the quinone head group. The changes at  $Q_A$  are observable at the earliest time point (50  $\mu$ s), while  $Q_B$  exhibits constant difference features (due to the presence of the reduced semiquinone) in all time points (see also *SI Appendix*, Fig. S5). Changes in both the  $Q_A$  and  $Q_B$  regions are much smaller at 200 ms. This is expected as the electron is transferred from  $Q_A$  to  $Q_B$  within several hundreds of microseconds and then after  $Q_B$  took up two redox equivalents it is replaced in the millisecond time scale with an oxidized quinone of the quinone pool present in the crystal.

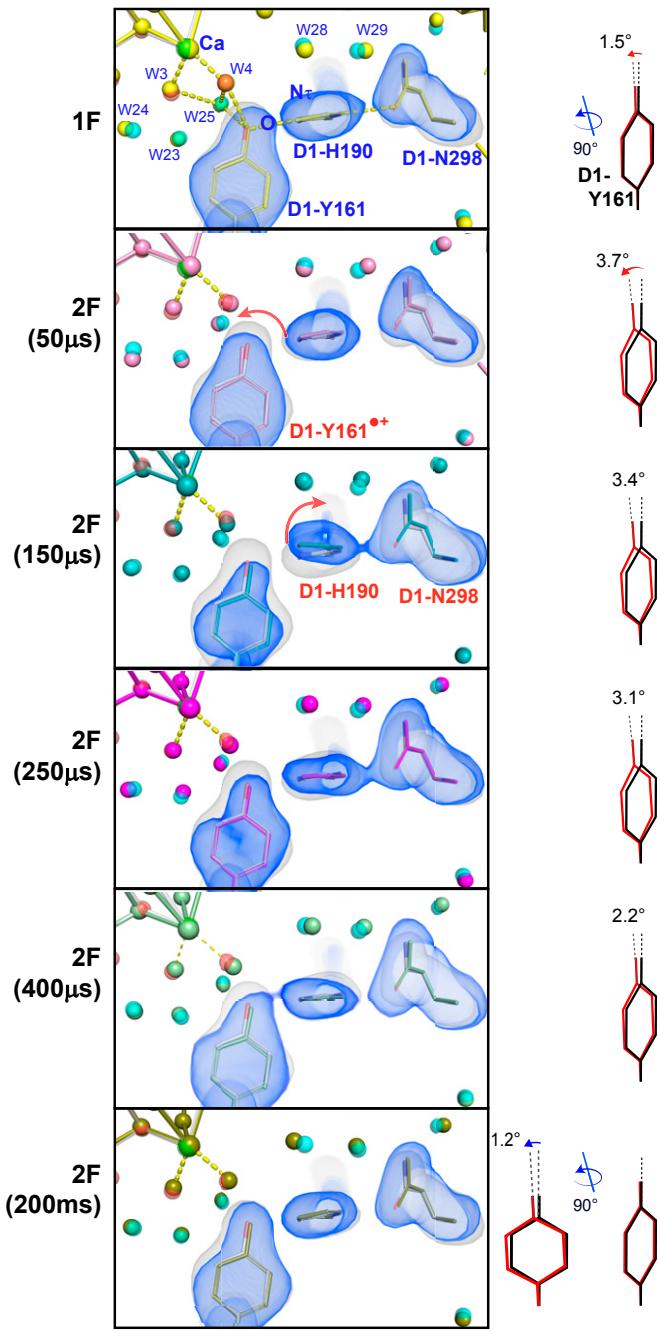
However, we cannot exclude an alternative scenario, where W4 is the proton relay. In both scenarios, moving of Glu189 away from both Ca and W25/W4 could allow its hydrogen-bonding interaction with the newly inserted  $O_X$  in the  $S_3$  state. The current data suggests that Ca could shuttle water molecules between the W3, W4/W25 positions, and the next water molecule. This will be discussed in more detail below.

In the isomorphous difference map (Fig. 4A and B), changes in the metal positions at the OEC are already noticeable (albeit weak) at 50  $\mu$ s and the metal distance changes can first be modeled in the 2Fo-Fc map at 150  $\mu$ s after the second flash; the elongation of the Mn1-Mn4 distance from ~4.8 to 5.2 Å (Fig. 5A) is observed as positive density appearing adjacent to Mn1 and Mn4 in Fig. 4. It suggests that a slight expansion (~0.4 Å) of the OEC occurs during the transition from the  $S_2$  to the  $S_3$  state. Interestingly, that expansion seems to reach its maximum value around 250 to 400  $\mu$ s and a small contraction is observed upon the completion of the  $S_3$  formation. We reported in our previous study that the additional oxygen ( $O_X$ , as oxo or hydroxo) is inserted as a bridging oxygen between Mn1 and Ca (20). Following the Mn1-Mn4 elongation, the  $O_X$  density starts to appear as a positive feature in the isomorphous difference electron density (Fig. 4A and B) between O5 and Mn1 at 150  $\mu$ s. Likewise, in the omit map (Fig. 4C), the presence of  $O_X$  becomes visible at the 150  $\mu$ s time point with ~30% peak height (Fig. 5C), and its intensity reaches nearly maximum around 400  $\mu$ s at a normalized level of about 60 to 70% of the value obtained for omitting O2 from the OEC (*Methods*), which corresponds to the calculated  $S_3$  state population in this state (*SI Appendix*, Table S2). At this time point, the OEC structure is very similar to that of the stable intermediate  $S_3$  state, indicating that in most of the PS II centers the changes at the OEC are complete around this time point. As discussed later, the  $O_X$  insertion kinetics matches favorably with

the Mn oxidation kinetics obtained from the  $K\beta_{1,3}$  XES (discussed below and see Fig. 5B and C).

The presence of a closed-cubane OEC structure has been proposed in the water insertion process during the  $S_2 \rightarrow S_3$  transition referred to as a carousel or pivot mechanism (36, 54). These proposals are based on the observation that there are two  $S_2$  states (high-spin and low-spin forms) often detected by electron paramagnetic resonance (EPR), and some theoretical studies predict their structures to have an open- and closed-cubane motif, respectively (25, 40) (see also *SI Appendix*, Fig. S1). In these mechanisms, the water insertion is accompanied by the rearrangement of the OEC at the open coordination site of Mn4 through the shift of O5 to form a closed-cubane motif, prior to the formation of the complete  $S_3$  form. This proposal leads to a flipping of bonds so that the original O5 ends up in the  $O_X$  position. Our current data, collected at pH 6.5 and room temperature, show that the Mn3-Mn4 distance remains constant at around 2.7 to 2.8 Å through the  $S_2 \rightarrow S_3$  transition (Fig. 5A), implying that the cluster maintains a di- $\mu$ -oxo configuration with the Mn4-O5-Mn3-O4 moiety anchored by the Glu333 residue forming a bidentate bridge to Mn3 and Mn4. Thus, we did not observe a closed cubane-like structure in the time-point data. The most straightforward interpretation of this is that the formation of such a closed-cubane structure is not necessary during the  $S_2 \rightarrow S_3$  transition at room temperature and neutral pH. We, however, note that we may not be able to detect such species, if 1) it is formed and decays before our first time point (50  $\mu$ s), 2) it is short-lived due to fast formation and decay kinetics, or 3) its fraction is for other reasons 10% or less at each time point. Recent theoretical studies have shown that both forms of the  $S_2$  state structure, the high- and low-spin forms, can be rationalized by an open-cubane structure (37, 55).

While the Mn3-Mn4 distance remains constant, the Mn4-Glu333 distance changes noticeably (Fig. 5A). It shortens by ~0.2



**Fig. 3.** Fo-Fc electron density omit map of the O6F data (gray) and model (gray) overlaid with the Fo-Fc omit maps (blue) and the models for 1F (yellow) and 2F time points (50  $\mu$ s: pink; 150  $\mu$ s: cyan; 250  $\mu$ s: magenta; 400  $\mu$ s: green; 200 ms: olive) in the region of D1-Tyr161 ( $Y_z$ ). D1-Tyr161, D1-His190, and D1-Asn298 (which were omitted for map generation) are shown together with the Ca of the Mn<sub>4</sub>Ca cluster. The tilt angle of the Tyr phenol ring with respect to the O6F structure (black) is shown at the right for each state (red). Within 50  $\mu$ s the electron is transferred from  $Y_z$  to  $P_{680}^{+*}$  as seen by the change in electron density around  $Y_z$ . At 150  $\mu$ s,  $Y_z$ , D1-His190, and D1-Asn298 all move away from the Mn<sub>4</sub>Ca cluster, indicating H-bonding changes that could be connected to proton transfer processes. In the 250- $\mu$ s data, D1-His190 and D1-Asn298 are back in their original positions and from there onward  $Y_z/Y_z^{\text{ox}}$  slowly returns to the position prior to the light flash, reflecting the reduction of  $Y_z^{\text{ox}}$  by the Mn<sub>4</sub>Ca cluster.

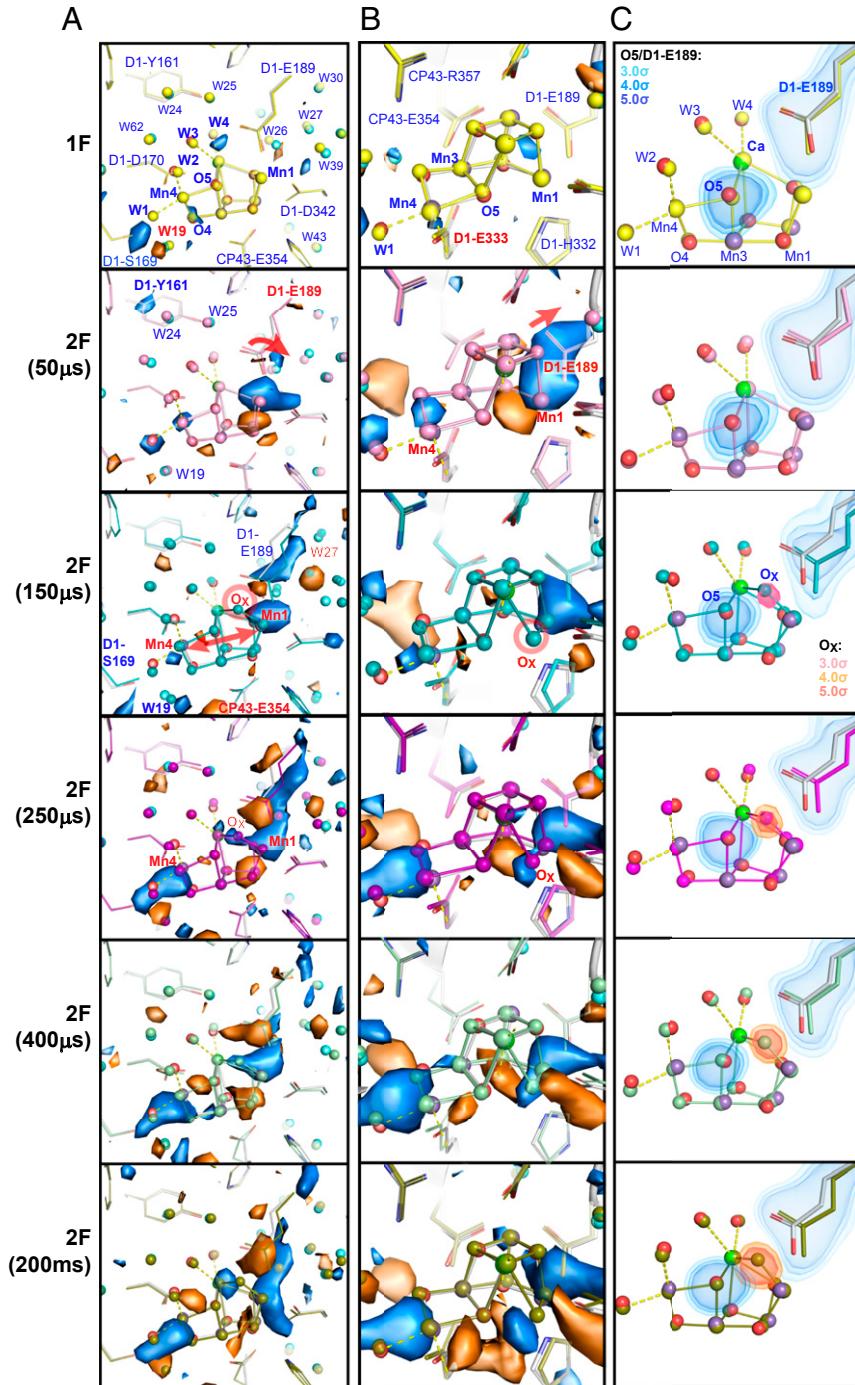
$\text{\AA}$  between O6F and 1F, likely reflecting the oxidation of Mn4 from Mn(III) to Mn(IV) upon the S<sub>1</sub>  $\rightarrow$  S<sub>2</sub> transition. During the transient states between the S<sub>2</sub> and S<sub>3</sub> states, the Mn4–Glu333

distance increases again from 1.8  $\text{\AA}$  to 2.2  $\text{\AA}$  and then shortens to be  $\sim$ 1.9  $\text{\AA}$  in the stable S<sub>3</sub> state. Similarly, the elongation of Ca–W3 and Mn4–W2 distances is visible slightly above the noise level. The variation in the Mn4–Glu333 distance could be related to the water-insertion event (*SI Appendix*, Fig. S1), either from the Ca-bound W3 site or from the Mn4-bound W2 site via O5 to O<sub>x</sub>. Another cause for the change in this distance could be that the movement of the Mn4–Mn3 moiety (necessary for the expansion of the cluster) precedes a change of the Glu333 position and that Glu333 is relaxing into its new position with some delay. Although we cannot exclude other processes happening around Mn4, an involvement of waters bound to Mn4 in the O<sub>x</sub> insertion would require the presence of a closed-cubane structure as an intermediate. As our data do not support the presence of such a closed-cubane structure in any of the time points, other water insertion routes seem more likely (discussed below).

Another important aspect of the OEC structure during the S<sub>2</sub>  $\rightarrow$  S<sub>3</sub> transition is the nature of the inserted O<sub>x</sub> (O6 in ref. 21), that is, whether O<sub>x</sub> is protonated or not, and the nature of its interaction to O5 that is located in its vicinity. The O5–O<sub>x</sub> distance or Mn1–O<sub>x</sub> distance becomes detectable in the refined structure at 150  $\mu$ s after the second flash, and the O5–O<sub>x</sub> and Mn1–O<sub>x</sub> distances were found to be  $1.9 \pm 0.14$   $\text{\AA}$  and  $1.7 \pm 0.25$   $\text{\AA}$ , respectively. In the final S<sub>3</sub> form, the distances are  $2.2 \pm 0.25$   $\text{\AA}$  for O5–O<sub>x</sub> and  $1.8 \pm 0.18$   $\text{\AA}$  for Mn1–O<sub>x</sub>. Error bars on these distances given here and displayed in Fig. 5A were calculated by generating 100 perturbed sets of structure factors for each structure using END/RAPID (56), refining the structural model separately against these, and calculating the SD for each bond of interest from the ensemble of structures obtained (details in *SI Appendix*). While the observed distances seem to indicate a transient shortening of Mn1–O<sub>x</sub> and elongation of O5–O<sub>x</sub> during the formation of S<sub>3</sub> (Fig. 5A), we cautiously state that within the error at the current resolution one cannot be conclusive about the changes. However, one can speculate that these distance changes might reflect changes of the protonation state at the O<sub>x</sub> site after the water insertion and the possible subsequent proton transfer processes described above. Interestingly, an elongation of the distance between W25 and Glu189 is visible 150 to 250  $\mu$ s after the second flash (*SI Appendix*, Fig. S6), indicating a weakening of the H-bonding interaction between them. This change could be coupled to the formation of a H-bonding interaction between O<sub>x</sub> and Glu189.

Suga et al. (19) initially reported 1.45  $\text{\AA}$  for the O6(x)–O5 distance and proposed a peroxide intermediate, which requires Mn reduction. This is not consistent with the Mn oxidation state assignment in the S<sub>3</sub> state (Fig. 1B) (57). Our recent study (20) on all of the S-state intermediates at  $\sim$ 2  $\text{\AA}$  resolution at room temperature reported 2.0 to 2.1  $\text{\AA}$  for the O5–O<sub>x</sub> distance in the S<sub>3</sub> state, and the changes as a function of time between S<sub>2</sub> and S<sub>3</sub> from this study (as described above) rule out the presence of a peroxide intermediate at any point in the S<sub>2</sub>  $\rightarrow$  S<sub>3</sub> transition or the S<sub>3</sub> state. Suga et al. have recently revised their distance estimates to 1.9  $\text{\AA}$  (21), based on data from frozen crystals. As described in the previous paragraph, a detailed estimation of the individual error for each atomic distance is necessary. The 1.9  $\text{\AA}$  distance reported in ref. 21 falls at the lower end of our range for the O5–O<sub>x</sub> distance.

In order to evaluate if differences in beam conditions (especially pulse length and beam size) could have an influence on the data reported, we collected data at the SPring-8 Ångstrom Compact free-electron LAser (SACLA) (58, 59) (see *SI Appendix* for details) in addition to our measurements at the Macromolecular Femtosecond Crystallography instrument (60) at the Linac Coherent Light Source at SLAC (LCLS) (61). XES and X-ray diffraction data were collected at both facilities with the difference that the pulse length was 7 fs at a beam size of 2  $\mu\text{m} \times 2.5 \mu\text{m}$  and the pulse energy was 0.3 mJ at SACLA, instead of 35 fs and 2 mJ and a beam diameter of  $\sim$ 3  $\mu\text{m}$  at LCLS. The obtained 2F (S<sub>3</sub>)

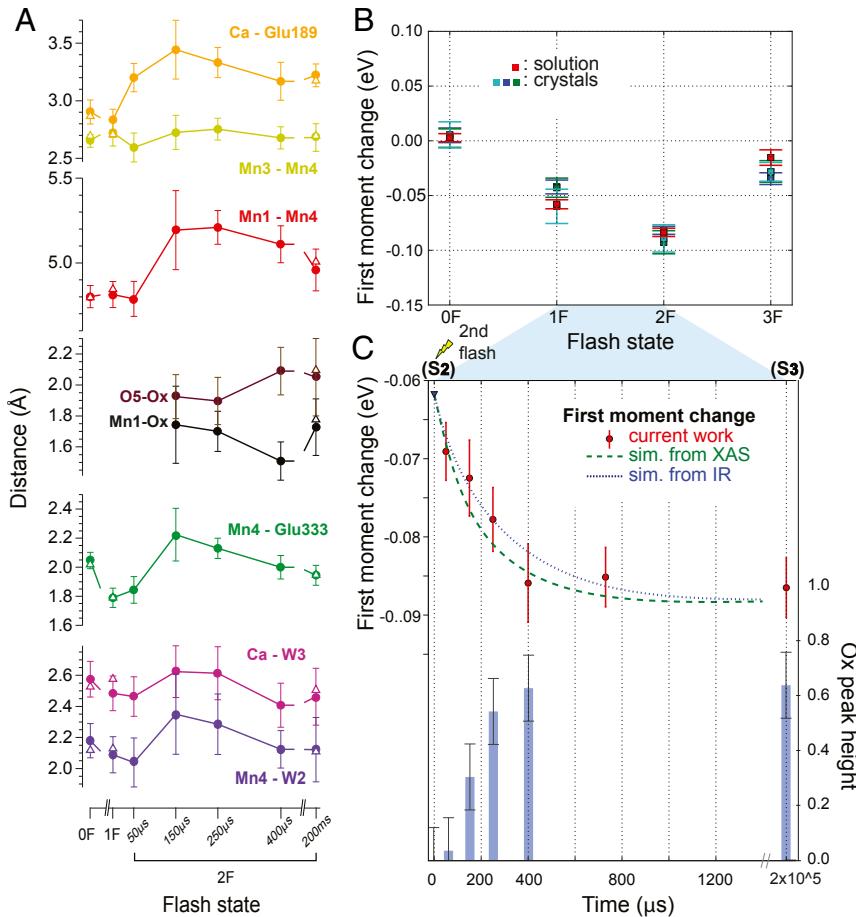


**Fig. 4.** Structural changes at the OEC in the  $S_2 \rightarrow S_3$  transition. (A and B) Fo-Fo isomorphous difference maps ( $\pm 3 \sigma$  in blue and orange) of 1F-0F and 2F(time point)-0F datasets in the region of the OEC in two different views for monomer I. (C) Fo-Fc omit maps obtained upon separately omitting D1-Glu189 (blue), O5 (blue), and  $O_x$  (red), respectively. The dark-state structure is shown as a ball and stick model (carbons in gray, nitrogens in blue, oxygens in red, unbound waters in cyan) overlaid with refined models for various illumination conditions indicated in the left margin. The 1F-0F ( $S_2-S_1$ ) isomorphous difference map does not feature any significant changes at the OEC (except the disappearance of W20, not shown here but see ref. 20, and the concomitant change in position for W19). At 50  $\mu$ s the movement of Glu189 away from Ca is seen, followed by Mn1-Mn4 moving apart and  $O_x$  binding between Mn1 and Ca from 150  $\mu$ s onward. The omit maps show the shift of Glu189 starting at 50  $\mu$ s and more dominantly at 150  $\mu$ s, while the  $O_x$  density appears in the maps starting at 150  $\mu$ s. While there are slight differences in the two PS II monomer sites (monomer I and II), the overall trend is the same on both sites.

structure from SACLA was refined to 2.4 Å resolution and isomorphous difference densities for the 2F-0F data showed features similar to the ones obtained from data collected at LCLS (SI Appendix, Fig. S7A). Likewise, the XES data obtained for the 2F state are similar to data collected at LCLS (SI Appendix, Fig. S7B). From this comparison, we conclude that the different X-ray parameters do not lead to differences in the reported data within the noise level of these measurements and hence cannot be the cause for differences in the distances (19, 21).

**Kinetics of the Mn Oxidation State Changes during the  $S_2 \rightarrow S_3$  Transition.** To investigate the correlation of the  $O_x$  insertion between Mn1 and Ca with the Mn(III) to Mn(IV) oxidation state

changes of Mn1 during the  $S_2 \rightarrow S_3$  transition, we analyzed the first-moment changes of the XES  $K\beta_{1,3}$  peak (Fig. 5B and C and SI Appendix, Fig. S8). Fig. 5B shows the Mn  $K\beta_{1,3}$  XES first moment of crystal and solution samples at each flash state. Within the error, the XES first moments of the crystal and solution samples behave similarly, confirming that the efficiencies of the S-state transition are comparable between both sample types. The change in the  $K\beta_{1,3}$  first-moment energy during the  $S_2 \rightarrow S_3$  transition is shown for solution samples in Fig. 5C. If single exponential kinetics are assumed, the time constant,  $\tau$ , of the oxidation state changes under the current experimental condition is  $\sim 350 \mu$ s (SI Appendix, Fig. S9).



**Fig. 5.** Changes during the  $S_2 \rightarrow S_3$  transition. (A) Distance changes for selected bond lengths of 0F, 1F, 2F, and the time points between 1F and 2F, with error bars estimated by generating 100 randomly perturbed datasets, re-refining, and reporting the resulting SD, as described in *SI Appendix*. Open triangles represent values obtained in our previous study for the  $S_1$  (0F),  $S_2$  (1F), and  $S_3$  (2F 200 ms) states (20); values are for monomer I. (B)  $K\beta_{1,3}$  Mn XES first moment changes of PS II crystals (three independent experiments) and solutions at each flash. All datasets are shifted to overlap for the first moment of the  $S_1$  state. (C)  $K\beta_{1,3}$  Mn XES first moment changes (red circles) after the second flash. The error bars (red vertical lines) for the XES data are estimated from the standard distribution of 1,000 iterations of randomly dividing all spectra in a dataset into two groups and calculating first moments for each group (75). The S-state populations estimated for our PS II solution samples (*SI Appendix*, Table S2) were taken into account to generate the dashed and dotted lines that show the predicted first moment shift based on time constants from IR (at 10 °C, with *T. elongatus*; ref. 29) and XAS (at room temperature with spinach; ref. 62) studies, respectively (*SI Appendix*). In the same panel, the increase of the  $O_x$  omit density in the crystal structure is shown as a histogram (blue bars). To obtain error bars for the  $O_x$  omit density, oxygen atom O2 was omitted from the OEC structure, and the SD of the O2 omit density over all datasets in both monomers was used for estimation.

The first-moment trend obtained from the current experiment falls well within the range of the kinetic data reported from time-resolved infrared (IR) and time-dependent X-ray absorption spectroscopy (XAS) studies (29, 62). The expected kinetic traces based on time constants obtained from the IR and XAS studies using our experimentally determined miss and double-hit parameters and S-state population for the 1F solution samples (*SI Appendix* and *SI Appendix*, Table S2 and Fig. S9) are also shown in Fig. 5C. We note that the degree of uncertainty in the IR and the XAS data has not been reflected in the simulated lines. Also, the differences among the three experiments, if any, would arise from the differences in the species (plants vs. thermophilic cyanobacteria), temperature (room temperature vs. 10 °C), and the sensitivity of each method to a certain part of the enzyme (metal vs. ligands).

Using IR measurements (at 10 °C, with *Thermosynechococcus elongatus*), Sakamoto et al. (29) described the  $S_2 \rightarrow S_3$  process with three phases with time constants:  $\tau_1 = 13 \mu s$ ,  $\tau_2 = 104 \mu s$ , and  $\tau_3 = 352 \mu s$  (see blue dotted line in Fig. 5C for a plot using only  $\tau_2$  and  $\tau_3$ ). They interpreted the first phase to reflect changes in the H-bond interaction of the  $Y_Z^{\bullet}$  with a  $COO^-$  group, resulting in a lag phase before initiation of  $Y_Z^{\bullet}$  reduction, while the second phase was assigned to changes in the water network and the  $COO^-$  groups around the  $Mn_4Ca$  cluster due to the rearrangement of the water molecules interacting with  $Y_Z^{\bullet}$ . The third phase was proposed to reflect the proton release coupled with oxidation of the  $Mn_4Ca$  cluster. In the second phase, they suggested that the W3 water moves to the open coordination site of Mn4 or Mn1 in the  $S_2$  state, which could be coupled with internal proton transfer. The XAS study by Zaharieva et al. (62) (at room temperature with spinach), which directly probes Mn, showed that

the  $S_2 \rightarrow S_3$  process can be fit with two time constants, a very fast transition with a time constant of  $\sim 26 \mu s$  ( $\tau_1$ ) followed by a slow one ( $\tau_2$ ) with  $317 \mu s$  (see green dashed line in Fig. 5C). Based on the kinetic H/D isotope effect, it was proposed that the fast phase of the  $S_2 \rightarrow S_3$  transition is connected to proton release to the bulk and the  $\sim 300\text{-}\mu s$  phase involves a proton-coupled electron transfer and oxidation of the  $Mn_4Ca$  cluster.

The oxidation state change from our XES data and those derived from the earlier IR and XAS experiments nearly coincide with the increasing intensity of  $O_x$  from crystallography during the  $S_2 \rightarrow S_3$  transition shown as a histogram in Fig. 5C. This indicates that the kinetics of water insertion and Mn oxidation are highly correlated within the error limits of our current data (*SI Appendix*, *Supplementary Discussion* and Fig. S9). In order to resolve the question of “water binding first” or “Mn oxidation first,” XES and diffraction data from earlier time points within the  $S_2 \rightarrow S_3$  transition with better signal/noise level will be required.

**Water Dynamics during the  $S_2 \rightarrow S_3$  Transition.** In the current and recent (20) room-temperature crystal structures at 2.01 to 2.27 Å resolution, we observe  $\sim 1,000$  waters in each PS II monomer. The fact that these waters have electron density in the XFEL crystal structures at each state obtained at room temperature implies that they are structurally and functionally important for PS II. Our current results identified regions in the vicinity of the OEC where waters are less and more mobile based on the magnitude of observed positional changes of these waters between different illumination states (Fig. 6). We hypothesize that the former region (channel O4, channel Cl1) is important for proton release, and the latter (channel O1) may play a role in substrate water intake.

As reported earlier by Kern et al. (20), electron density of W20 disappears in the  $S_2$  state. When present in  $S_1$ , this water is located close to the OEC in channel O4. We found that W20 is not visible in any of the structures measured during the  $S_2 \rightarrow S_3$  transition. As W20 is already absent in the  $S_2$  state it is not likely that W20 is the source of the substrate water that binds to the  $O_X$  position in the  $S_2 \rightarrow S_3$  transition, contrary to some proposals (e.g., ref. 54 and *SI Appendix*, Fig. S1). Alternatively it was proposed that a change in the O4 channel involving W20 is connected to its role as a proton release path and that this channel could be active in the  $S_0 \rightarrow S_1$  transition but not in the  $S_2 \rightarrow S_3$  or  $S_3 \rightarrow S_0$  transitions (20, 63). W19, located in the vicinity of W20 in the dark state ( $S_1$ ), shows position changes between O4 and D1-S169. The shortest distance between W19 and O4 (2.4 Å) was observed in the  $S_2$  state (1F). The displacement of W19 between the 0F and 1F states appears as a negative electron density in the isomorphous difference maps (Fig. 4), indicating a large shift in its position from the dark state (0F), corresponding to the shift of ~0.4 Å observed in the refined coordinates. We speculate that W19 takes a role of mediating proton movement between the OEC (O4) and the D61, S169, and R357 side chains, either for releasing protons from the OEC or compensating charges to avoid charging of the OEC.

Unlike waters in the O4 channel or the C11 channel, which have also been proposed as water channels (64–66), the waters along the O1 channel show significant positional changes during the  $S_2 \rightarrow S_3$  transition (Fig. 6). A ring of five waters, W26–30 near O1 of the OEC located at the end of the O1 channel (Fig. 6 and *SI Appendix*, Fig. S10), could serve as an entrance for substrate water, shuffling water like a “water wheel” to the OEC. This idea is supported by the observation that concomitant to the  $O_X$  appearance, a significant negative electron density is seen near W27 during 150 to 400 μs after the second flash (Fig. 4A). Alternatively, the changes in water positions in this “water wheel” could play a role in charge compensation during the S-state advancement as suggested previously (20).

Recently Suga et al. (21) reported the appearance of a new water next to residue CP43-Val410 in their cryogenic 2F data in the region of the O1 channel. In our previous (20) and current room-temperature data we do not observe the occurrence of new water molecules in this region. Instead, the same region of electron density is explained by a double conformation of the side chain of residue CP43-Glu413, which is present both in the dark and in all of the illuminated states. It should be noted that instead of the glycerol molecules modeled in the cryogenic data (21), we observed several additional water molecules in this area. Some of these waters (e.g., W76 and W77) and surrounding amino acid side chains (e.g., V-Lys47, V-Tyr137, and CP43-Glu413) exhibit strong variability in position between the different illumination states, indicating a mobile region of the water network, consistent with the idea of the O1 channel’s being an entrance for substrate water.

**Possible Routes for  $O_X$  Insertion.** Water insertion during the  $S_2 \rightarrow S_3$  transition can be clearly followed by the buildup of the  $O_X$  density between Ca and Mn1. However, the origin of  $O_X$ , that is, from where and how the water reaches the  $O_X$  site and its protonation state once inserted remain open questions. Despite the interesting dynamics visible in the “water wheel” region of the O1 channel (discussed above), it is difficult to identify an exact water insertion route. This may indicate that the energetic barrier for water insertion is higher than for water transport to the OEC. There are four pathways that have been proposed in the literature (see *SI Appendix*, Fig. S1 for cases 2, 3, and 4): 1)  $W_{N1}$  (non-OEC ligated water near Mn1)  $\rightarrow$  Mn1 (22), 2) W3  $\rightarrow$  Mn1 (28–30, 67), 3) W3  $\rightarrow$  Mn4 (14, 25, 28–30, 38, 68–71), or 4)  $W_{N2}$  (non-OEC ligated water near Mn4, for example W24 or W19)  $\rightarrow$  Mn4 (36, 39, 72). In 1 and 2, water directly goes into the Mn1 open coordination site ( $O_X$ ) and the OEC remains in the open-

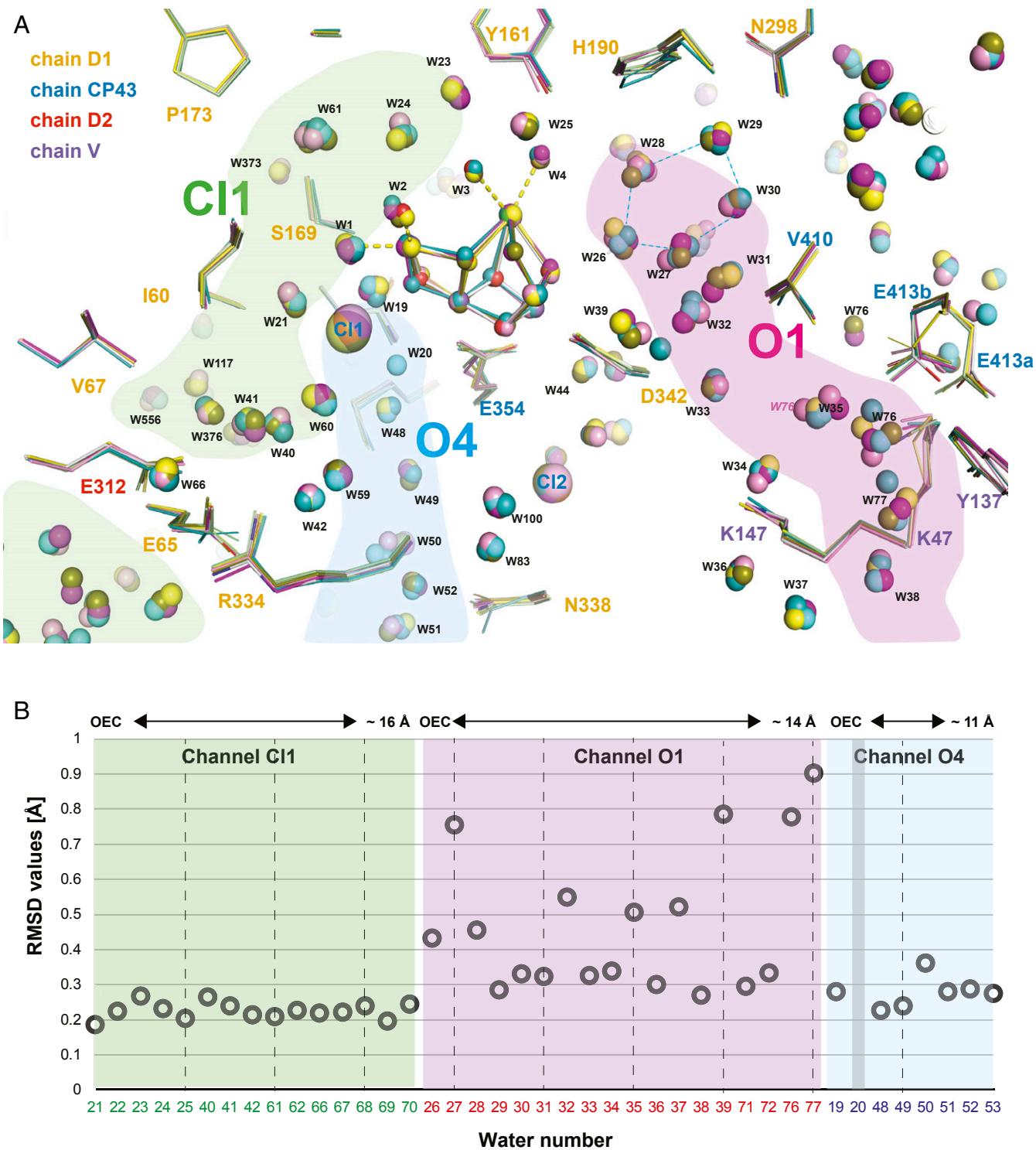
cubane configuration throughout the  $S_2 \rightarrow S_3$  transition. In 3 and 4, on the other hand, water first binds as a ligand to Mn4 by shifting O5 toward Mn1 (i.e., a closed-cubane configuration), and this water shifts to the O5 position through flipping of bonds, thus the original O5 ending up at the  $O_X$  position in  $S_3$  [pivot/carousel mechanism (36, 39, 72)].

Among the above four proposals, the  $W_{N1} \rightarrow$  Mn1 pathway (case 1) is often disfavored due to steric hindrance of water movement to Mn1 caused by Val185 (69), unless this residue rotates at the water insertion event (22, 37). The density of the Val185 is, however, well-defined throughout the time point data we collected in this study and does not show any large motion. For water insertion to Mn4 (cases 3 or 4), it requires that the closed-cubane-like configuration forms prior to the formation of the  $S_3$  state. As discussed above, the current results obtained by room temperature crystallography do not provide any evidence for the closed-cubane-like structure at any of the time points collected in this study. Based on the above observations, we hypothesize that water is inserted into the  $O_X$  site from W3 and refilled via the highly mobile waters in the O1 channel (case 2; see Fig. 7B). Chrysina et al. (35) recently reported an EPR signal that shows a small fraction of Mn(IV) in a highly distorted ligand environment in native PS II samples poised in the  $S_3$  state. This signal was assigned by the authors as arising from either a five-coordinate Mn1(IV) within an open-cubane configuration or a five-coordinate Mn4(IV) within a closed-cubane configuration. Our data demonstrate that, at all times tested, the population of the proposed closed-cubane state, if present, cannot exceed our detection limit of 10% during the  $S_2 \rightarrow S_3$  transition. Thus, we propose that a five-coordinate Mn1(IV) within an open-cubane conformation is a more likely intermediate during the  $S_2 \rightarrow S_3$  transition.

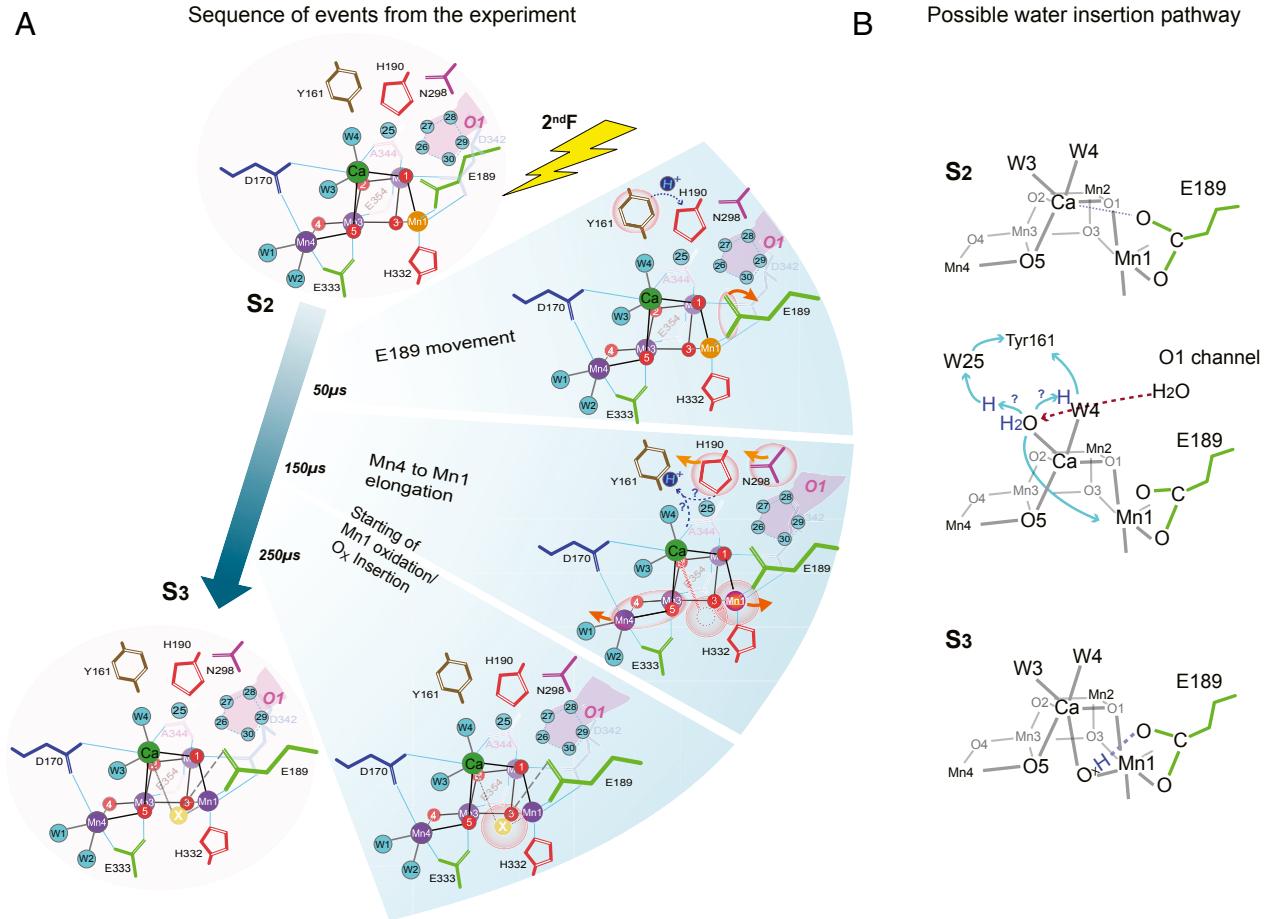
**Sequence of Events during the  $S_2 \rightarrow S_3$  Transition.** Fig. 7A summarizes the sequence of events that are observed around the OEC during the  $S_2 \rightarrow S_3$  transition, based on crystallography and XES data. We show that collecting snapshot data at various time points at room temperature using XFELs allows for delineating the sequence of events that determine the directionality of the reaction, proceeding from one stable intermediate state to another, and providing a clear rationale for each transition. This combined crystallography and spectroscopy study correlates the structural changes occurring in the protein with the redox changes taking place at the metal center.

The earliest event we observe in this time-resolved study (Fig. 7A, Top) is the motion of  $Y_Z$  located between the primary donor, P<sub>680</sub>, and the OEC, together with D1-His190-Asn298 residues, as well as the motion of D1-Glu189 next to Ca. This change occurs at <50 μs after the second light flash. These early changes can be explained by the transfer of an electron from  $Y_Z$  ( $Y_Z \rightarrow Y_Z^{\bullet+}$ ) to P<sub>680</sub><sup>•+</sup> immediately after the charge separation between P<sub>680</sub><sup>\*</sup> (excited state of P<sub>680</sub>) and pheophytin. The oxidation of  $Y_Z$  is expected to change the H-bonding network along the residues D1-Y<sub>Z</sub>-His190-Asn298 and the surrounding waters in their vicinity, while this process may also serve as a proton release pathway. Although we do not resolve it in this study at 50 μs, the changes at Glu189 will likely be preceded by the changes around  $Y_Z$ .  $Y_Z$  is located about 4 Å away from Glu189 and the formation of the positive charge at the  $Y_Z$ /His190 pair that precedes oxidation of the OEC could trigger structural changes in their surroundings, inducing a shift of Glu189 away from Ca as observed in the 2F(50 μs) data.

In the next step (<150 μs after the second flash; Fig. 7A, Middle), the elongation of the Mn1-Mn4 distance is observed, while the Mn3-Mn4 pair maintains the di-μ-oxo bridged structure. We hypothesize that the proton release around  $Y_Z$  triggers the shift of the Mn4 and Mn1 positions in the early stage of the  $S_2 \rightarrow S_3$  transition, which seems to be completed by ~150 μs. Subsequent to the Mn1-Mn4 motion,  $O_X$  becomes gradually



**Fig. 6.** The water network in the neighborhood of the OEC. (A) The structure of the protein and refined positions of waters surrounding the Mn<sub>4</sub>Ca cluster are shown for monomer I for all time points [0F: gray; 1F: yellow; 2F(50 µs): pink; 2F(150 µs): cyan; 2F(250 µs): magenta; 2F(400 µs): green; 2F(200 ms): olive]. Residues are labeled according to subunit with D1 yellow, D2 red, CP43 blue, and PsbV magenta. Proposed channels connecting the OEC to the solvent-exposed surface of PS II for water movement or proton transfer are indicated in green, light blue, and pink. Oscillation of the cluster of five waters (W26–W30) in the proximity of O1 of the OEC is visible. (B) The rmsds of water positions (average over both monomers and all time points) within the vicinity of the Mn<sub>4</sub>Ca cluster are shown as a function of their distance from the Mn<sub>4</sub>Ca cluster. The waters in the O1 channel are more mobile on average than the waters in the C1 and O4 channels. As water 20 is only present in the 0F state but absent in the 1F and 2F states no rmsd for its position can be given. W39 is only present in one monomer and hence only the average over one monomer is given.



**Fig. 7.** Schematic of the  $S_2 \rightarrow S_3$  transition. (A) The sequence of events in time leading to the insertion of  $O_x$  between Mn1 and Ca. Mn1 oxidation from (III) to (IV) is shown as a color change from orange to purple. The other Mn atoms are all in oxidation state (IV) and are shown in purple. Ca is shown in green. The ligands of Mn and Ca,  $Y_z$ , and other neighboring residues and the water ligands of Mn4 and Ca are shown. Possible pathways for proton transfer are depicted as well. (B) Schematic showing possible water insertion from the O1 channel via W4/W3 to the  $O_x$  site and proton movements from W3 to  $Y_z$  via W25 or W4. The protonated oxo bridge at the  $O_x$  position could form a hydrogen bond with the carboxylate chain of Glu189.

visible in the electron density maps; it is still within the noise level at 50  $\mu$ s, but it is at ~30% of its final occupancy by 150  $\mu$ s, and ~80% by 250  $\mu$ s, and close to full occupancy by 400  $\mu$ s (Fig. 7B, Bottom). The oxidation of Mn1 appears to be directly coupled to the insertion of water,  $O_x$ , at the Mn1 open-coordination site into a bridging position between Ca and Mn1. Our previous XFEL data (20) as well as the current time-resolved data are consistent with this configuration.

How  $O_x$  arrives as a ligand to Mn1 in the  $S_3$  state and what its protonation state is remain speculative at this point. However, we propose a hypothesis in Fig. 7B based on the OEC structural changes and the water and ligand motions experimentally observed in this study (Fig. 7A), as well as based on other experimental evidence reported in the literature. In the current study, we neither detected a closed-cubane-like structure nor a change in the Mn3–Mn4 moiety, both features that may be necessary prerequisites for indicating a water ( $O_x$ ) insertion pathway that involves Mn4. Therefore, we hypothesize that  $O_x$  does not originate from a ligand of Mn4 but is derived from the Ca-bound water W3.

Several studies have proposed that Ca-bound water (W3) may serve as the entrance point for substrate water (see, e.g., refs. 28, 30, 54, and 73). For example, FTIR studies showed changes in water vibrational modes during the  $S_2 \rightarrow S_3$  transition that are sensitive to the substitution of Ca with Sr (14, 28, 68, 70), and these modes were assigned to Ca-bound W3. We also proposed in our recent study that W3 could serve as an entry point for

substrate water (20). The high mobility of waters in the O1 channel, especially in the region of the “water wheel” observed in this study (Figs. 6 and 7A), supports the hypothesis that the substrate water likely arrives from the O1 channel. The pathway of water from the “water wheel” to W3 is likely via W4 (Fig. 7B, Middle). If the insertion of the water occurs from the W3 site, this could be triggered by the oxidation of  $Y_z$  and the proton transfer to the neighboring His190 ( $Y_z^{ox}$ –His190 $^+$  formation), because it reduces the  $pK_a$  of W3. Then, upon oxidation of the Mn cluster [five coordinate Mn1(III)  $\rightarrow$  five coordinate Mn1(IV)] and reduction of  $Y_z^{ox}$ , the tyrosine could accept the proton indirectly (e.g., via W4 or W25) from W3, leading to formation of a hydroxide that is concomitantly transferred to the open coordination site of Mn1(IV) (30). In the current study, we show that the oxidation of Mn and water ( $O_x$ ) binding are highly correlated events, but we cannot distinguish the exact order of these two events within the current time resolution and signal-to-noise ratio of XES and crystallography data. The noticeable shift in the first moment of the X-ray emission spectra at 50  $\mu$ s after the second flash (Fig. 5C and SI Appendix, Fig. S8) could suggest that Mn oxidation triggers  $O_x$  insertion, although the data are also compatible with a faster structural rearrangement process that does not change the oxidation state of the Mn<sub>4</sub>Ca cluster followed by concomitant Mn oxidation and  $O_x$  insertion. Most theoretical studies have proposed a concerted mechanism that requires binding of water first before Mn oxidation. However, recently Chrysina

et al. (35), based on W-band EPR results, proposed oxidation of Mn1 prior to binding of water, which would also be consistent with our results presented above.

It has been proposed that an O<sub>5</sub>–O<sub>X</sub> (or O<sub>6</sub>) peroxy-bond may form already in the S<sub>3</sub> state (19). We clearly showed previously and confirm in the current study that no O<sub>5</sub>–O<sub>X</sub> bond is formed in the S<sub>3</sub> state or any of the time points during the S<sub>2</sub> → S<sub>3</sub> transition. Moreover, our XES results do not show a reduction of Mn in the S<sub>3</sub> state, that should accompany any formation of an O–O peroxy-bond. Instead, our data clearly show that O<sub>X</sub> is a bridging ligand between Ca and Mn1, and we propose that O<sub>X</sub> is hydrogen-bonded to the carboxylate oxygen of Glu189 located at a distance of 2.4 Å (Fig. 7B, Bottom). We note that O<sub>X</sub> has been proposed as a hydroxide ligand based on EPR data and theoretical studies (23, 37, 40, 67), satisfying the S = 3 spin state configuration assigned for the S<sub>3</sub> state.

The current study demonstrates that untangling the structural sequence of events during the transition from one intermediate state to another in a time-resolved manner provides mechanistic insights into how the photochemically induced reactions proceed at the donor and acceptor sides. These studies take us one step closer to understanding the even more complex and highly orchestrated chemical process that is expected during the S<sub>3</sub> → S<sub>0</sub> transition in PS II. In this transition the catalytic center is first oxidized one more step by the absorption of another photon, which is followed by a cascade of events that includes the release of two protons the O–O bond formation, release of O<sub>2</sub> and the binding of one water, resetting the chemistry for the next catalytic cycle.

## Methods

Dimeric PS II was extracted and purified from *T. elongatus* and crystallized as described previously (18, 20). Activity and intactness of the samples used was confirmed by O<sub>2</sub> evolution measurements using a Clark-type electrode, by EPR measurements to assay for Mn(II), and by membrane inlet mass spectrometric measurements of O<sub>2</sub> production. Based on these measurements the S-state population for different illumination states was determined (*SI Appendix, Table S2*). Acoustic droplet ejection together with a drop on tape setup (74) was utilized to perform SFX and XES experiments at both the LCLS and SACLA XFEL facilities (59, 60). Illumination of samples was achieved using nanosecond-length pulses at 532/527 nm from Nd:YAG or Nd:YLF lasers with timing of 0.2 s between individual illumination pulses for generation of steady states and variable timing (50 to 400 µs), between the final illumination and X-ray probing for 2F(time point) data collection. SFX data

were collected using a Rayonix MX340-XFEL or an octal MPCCD detector. XES signal was collected by means of an 16 analyzer crystal array in van Hamos geometry together with an ePIX 100 or a single module MPCCD detector (75). SFX data processing used the cctbx.xfel software framework (41, 76). Crystallographic model building, refinement and map calculation was performed with Phenix (77) and Coot (78) and figures were generated using PyMol (79). END/RAPID (56) was used to derive error estimates for distances. Detailed protocols can be found in *SI Appendix, Methods*.

**Data Availability.** X-ray diffraction datasets and associated models have been deposited in the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (<https://www.rcsb.org>) under PDB ID codes 6W10 for the 0F, 6W1P for the 1F, 6W1Q for the 2F(50 µs), 6W1R for the 2F(150 µs), 6W1T for the 2F(250 µs), 6W1U for the 2F(400 µs), and 6W1V for the 2F(200 ms) data.

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