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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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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, S1, S2, S3, and S0, showing that a water molecule is inserted during the S2 → S3 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 O2 formation, we recorded diffraction and Mn X-ray emission spectroscopy (XES) data at several time points during the S2 → S3 transition. At the electron acceptor site, changes due to the two-electron redox chemistry at the quinones, QA and QB, are observed. At the donor site, tyrosine Y71 and His190 H-bonded to it move by 50 µs after the second flash, and Glu189 moves away from Ca. This is followed by Mn1 and Mn4 moving apart, and the insertion of O2(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 (r of ~350 µs) during the S2 → S3 transition mirrors the appearance of O2 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

Dioxygen, 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. L4), which contains a heteronuclear, oxo-bridged Mn4Ca cluster that catalyzes the reaction:

$$2\text{H}_2\text{O} \rightarrow \text{O}_2 + 4\text{e}^- + 4\text{H}^+$$.  

To allow this reaction to take place, the OEC accumulates four oxidizing equivalents, stepping through intermediates that are referred to as the S-states (S0, i = 0 through 4) (Fig. 1B) (1, 2). These oxidation reactions are driven by the light-induced charge separations in the reaction center of PS II that comprises the chlorophyll α moiety P680 (most likely consisting of the four excitonically coupled chlorophylls P681, P682, ChlP, and ChlC2) and the neighboring pheophytin molecules PheoD1/D2 as well as the two plastoquinones QA and QB. The one-electron photochemistry at

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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 codes 6W1O 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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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 YZ. Once four oxidizing equivalents are accumulated (S0-state), the formation of dioxygen occurs spontaneously and leads to the release of O2 and the reformation of the S0-state. At the electron acceptor side of PS II, the electrons created in the charge separation process are transferred to plastoquinone QA and subsequently to plastoquinone QB. After two light-induced charge separations, the dihydroplastoquinol, QBH2, forms a new plastoquinone molecule (Fig. 1 A and B) (3, 4).

The significance of the S2 → S3 transition among the four catalytic steps of the Mn4Ca 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− depletion (5, 6), substitution of Ca2+ by Sr2+ in the OEC (7, 8), low temperature (9), and a number of site-directed mutations (10). In addition, there is substantial evidence that the S2 → S3 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 conditions (10). In addition, the S2 and S3 states (Mn4+ yellow, Mn4+ purple, Ca2+ 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 (Qx = O2− or OH−) as a new bridging ligand between Mn1 and Ca (adapted from ref. 20).

The S2 → S3 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 (P680, Pheo) and stabilization with the acceptor quinones, QA and QB, toward the cytoplasm, and YZ and OEC with catalytic Mn4Ca 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 P680. The white and gray areas show the redox changes of the OEC (S1 to S0) and YZ together with the charge separation reactions, while the outer blue circle shows the concomitant acceptor side chemistry of the quinones. The S2 → S3 transition, marked by the red box, is discussed in detail in the main text. (C) The structures of the Mn4Ca cluster, showing the major changes between the S2 and S3 states (Mn3+ yellow, Mn4+ purple, Ca2+ 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 (Qx = O2− or OH−) as a new bridging ligand between Mn1 and Ca (adapted from ref. 20).
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 μs) during the S2 → S1 transition, probing the kinetics of the electronic and structural changes.

**Results and Discussion**

**Structural Changes during the S2 → S1 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 (S0), single-illuminated (S1), and doubly illuminated (S2) 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 μs after the second flash) during the S2 → S1 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 μs 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 μs and 250 μs) and the improved resolution.

**QA/Qb Acceptor Quinone Sites.** Although the plastoquinones QA and Qb have identical chemical structures, their binding constants inside PS II are quite different, due to their significantly different binding pockets. The binding energies of QA and Qb have been calculated to be −56.1 kcal/mol and −37.9 kcal/mol, respectively (42). The lower binding affinity of Qb 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 Qb compared to QA.

The second flash leads to the reduction of QA to Qa and subsequently to the Qa to Qb electron transfer, protonation of Qb', and the exchange of QbH2 with Qb. Qa' formation is clearly seen at 50 μs by the twist of Qa' relative to the QA position and the ~0.2 Å decrease of the H-bonding distances to D2-His214 and the backbone nitrogen of D2-Ph261 that is expected due to the increased electronegativity of the semiquinone groups. It is interesting that the isomorphous difference maps around QA/Qa' change much less than observed for Qb upon Qb' formation (Fig. 2). This suggests that the more rigid protein pocket around QA (showing a consistently smaller B-factor compared to the QB (Fig. 2). This suggests that the more rigid protein pocket around QA compared to that of Qb.

Since the QA to Qb and Qa' to Qb 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 μs and 400 μs (Fig. 2 and SI Appendix, Fig. S5) reflect the rearrangements of protein/H-bonding due to the reduction of QA and the onset of the electron transfer between QA' and Qb. The negligible differences observed in the 2F-0F isomorphous difference maps at the 200 ms time point at the QA-site show that the electron transfer from QA' to Qb and the exchange of QbH2 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 Y2 (D1-Tyr161)/His190 Site.** At the donor site the tyrosine residue, Y2 (D1-Tyr161), which is located between P680 and the OEC (Fig. L4), plays a critical role for electron transfer between these two sites (46–48). Fig. 3 shows the omit maps and models of the Y2, D1-His190, and D1-Asn298 residues, overlaid with the 0F(S1) omit map and structural model. Oxidation of Y2 after the charge separation at P680 has a half-life in the nanosecond to microsecond range (49–51). Therefore, small, but noticeable, changes in this area at 2F(50 μs) correspond to the changes induced by the oxidation of Y2 after electron transfer to P680 which is coupled to the transfer of the phenolic proton to the H-bonding partner His190 (see SI Appendix, Fig. S6). Glu189 moves away from W25 (2.6 Å). After the second flash, Y2 also moves away from W25 by ~0.3 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 S2 → S1 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 μs 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 Y2. One carboxylate oxygen of this residue ligates to Mn1, while the second carboxylate oxygen has a weak interaction with Ca in the S1(0F) and S2(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 ~2.8 (1F, S2) to ~3.2 Å [2F(50 μs)] (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 S2 → S3 transition. We observe that 150 μs after the second flash, Glu189 also moves away from W25 by ~0.3 Å. This is compatible with the time scale of Y2 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).
modeled in the 2Fo-Fc map at 150 weak) at 50 in the metal positions at the OEC are already noticeable (albeit Mn1 and Mn4 in Fig. 4. It suggests that a slight expansion (≈4 Å) of the OEC occurs during the transition from the S2 to the S3 (Fig. 5 – elongation of the Mn1 position, and the next water molecule. This will be discussed in more detail below.

In the isomorphous difference map (Fig. 4 A and B), changes in the metal positions at the OEC are already noticeable (albeit weak) at 50 μs and the metal distance changes can first be modeled in the 2Fo-Fc map at 150 μs 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 S2 to the S3 state. Interestingly, that expansion seems to reach its maximum value around 250 to 400 μs and a small contraction is observed upon the completion of the S3 formation. We reported in our previous study that the additional oxygen (OX, as oxo or hydroxo) is inserted as a bridging oxygen between Mn1 and Ca (20). Following the Mn1–Mn4 elongation, the OX density starts to appear as a positive feature in the isomorphous difference electron density (Fig. 4A and B) between O5 and Mn1 at 150 μs. Likewise, in the omit map (Fig. 4C), the presence of OX becomes visible at the 150 μs time point with ∼30% peak height (Fig. 5C), and its intensity reaches nearly maximum around 400 μs 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 S1 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 S1 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 OX insertion kinetics matches favorably with the Mn oxidation kinetics obtained from the Kp1,3 XES (discussed below and see Fig. 5 B and C).

The presence of a closed-cubane OEC structure has been proposed in the water insertion process during the S2 → S3 transition referred to as a carousel or pivot mechanism (36, 54). These proposals are based on the observation that there are two S2 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 S3 form. This proposal leads to a flipping of bonds so that the original O5 ends up in the OX 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 S2 → S3 transition (Fig. 5A), implying that the cluster maintains a di-μ-oxo configuration with 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 S2 → S3 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 μs), 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 S3 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
transient states between the S2 and S3 states, the Mn4 flash, reflecting the reduction of YZ (red). Within 50 μs after the second flash, D1-Tyr161 (YZ), D1-His190, and D1-Asn298 (which were omitted for map generation) are shown to-400 μs: pink; 250 μs: magenta; 150 μs: cyan; 200 μs: olive) in the region of D1-Tyr161 (YZ). D1-Tyr161, D1-His190, and D1-Asn298 (which were omitted for map generation) are shown to-3° after the second flash, and the O5–OX distance becomes detectable in the refined structure at 150 μs after the second flash, and the O5–OX and Mn4–OX distances were found to be 1.9 ± 0.14 Å and 1.7 ± 0.25 Å, respectively. In the final S3 form, the distances are 2.2 ± 0.25 Å for O5–OX and 1.8 ± 0.18 Å for Mn1–OX. 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–OX and elongation of O5–OX during the formation of S3 (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 OX 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 μs 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 OX and Glu189.

Suga et al. (19) initially reported 1.45 Å for the O6(χ1)–O5 distance and proposed a peroxy intermediate, which requires Mn reduction. This is not consistent with the Mn oxidation state assignment in the S3 state (Fig. 1B) (57). Our recent study (20) on all of the S-state intermediates at ∼2 Å resolution at room temperature reported 2.0 to 2.1 Å for the O5–OX distance in the S3 state, and the changes as a function of time between S2 and S3 from this study (as described above) rule out the presence of a peroxy intermediate at any point in the S2 → S3 transition or the S3 state. Suga et al. have recently revised their distance estimates to 1.9 Å (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 Å distance reported in ref. 21 falls at the lower end of our range for the O5–OX 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 (SACLX) (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 μm x 2.5 μm and the pulse energy was 0.3 mJ at SACLX, instead of 35 fs and 2 mJ and a beam diameter of ∼3 μm at LCLS. The obtained 2F (S3)

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Fig. 3. Fo-Fc electron density omit map of the 0F data (gray) and model (gray) overlaid with the Fo-Fc density omit map (blue) and the models for 1F (yellow) and 2F time intervals 50 μs (pink); 150 μs (cyan); 200 μs (magenta); 250 μs (green); 200 ms (olive). The tilt angle of the Tyr phenol ring with respect to the 0F structure (black) is shown at the right for each state (red). Within 50 μs the electron is transferred from YZ to S3** as seen by the change in electron density around YZ. At 150 μs, YZ, D1-His190, and D1-Asn298 all move away from the Mn4Ca cluster, indicating H-bonding changes that could be connected to proton transfer processes. In the 250-μs data, D1-His190 and D1-Asn298 are back in their original positions and from there onward YZ/YZ slow returns to the position prior to the light flash, reflecting the reduction of YZ** by the Mn4Ca cluster.

Å between 0F and 1F, likely reflecting the oxidation of Mn4 from Mn(II) to Mn(IV) upon the S1 → S2 transition. During the transient states between the S2 and S3 states, the Mn4–Glus33 distance increases again from 1.8 Å to 2.2 Å and then shortens to be ∼1.9 Å in the stable S3 state. Similarly, the elongation of Ca–W3 and Mn4–W2 distances is visible slightly above the noise level. The variation in the Mn4–Glus33 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 OX. 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 Glus33 position and that Glus33 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 OX 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 S2 → S3 transition is the nature of the inserted OX (O6 in ref. 21), that is, whether OX is protonated or not, and the nature of its interaction to OS that is located in its vicinity. The O5–OX distance or Mn1–OX distance becomes detectable in the refined structure at 150 μs after the second flash, and the O5–OX and Mn1–OX distances were found to be 1.9 ± 0.14 Å and 1.7 ± 0.25 Å, respectively. In the final S3 form, the distances are 2.2 ± 0.25 Å for O5–OX and 1.8 ± 0.18 Å for Mn1–OX. 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–OX and elongation of O5–OX during the formation of S3 (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 OX 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 μs 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 OX and Glu189.

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structure from SACLA was refined to 2.4 Å resolution and iso-
morphous 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 pa-
rameters 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 S2 → S3
Transition. To investigate the correlation of the OX insertion
between Mn1 and Ca with the Mn(III) to Mn(IV) oxidation state
changes of Mn1 during the S2 → S3 transition, we analyzed the
first-moment changes of the XES Kβ1,3 peak (Fig. 5B and C and
SI Appendix, Fig. S8). Fig. 5B shows the Mn Kβ1,3 XES first
moment of crystal and solution samples at each flash state.
Within the error, the XES first moments of the crystal and solu-
tion samples behave similarly, confirming that the efficiencies
of the S-state transition are comparable between both sample
types. The change in the Kβ1,3 first-moment energy during the
S2 → S3 transition is shown for solution samples in Fig. 5C. If
single exponential kinetics are assumed, the time constant, τ, of
the oxidation state changes under the current experimental condition
is ~350 μs (SI Appendix, Fig. S9).
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 S2 → S3 process with three phases with time constants: τ1 = 13 μs, τ2 = 104 μs, and τ3 = 352 μs (see blue dotted line in Fig. 5C for a plot using only τ2 and τ3). They interpreted the first phase to reflect changes in the H-bond interaction of the YZ with a COO− group, resulting in a lag phase before initiation of YZ reduction, while the second phase was assigned to changes in the water network and the COO− groups around the Mn4Ca cluster due to the rearrangement of the water molecules interacting with YZ. The third phase was proposed to reflect the proton release coupled with oxidation of the Mn4Ca cluster. In the second phase, they suggested that the W3 water moves to the open coordination site of Mn4 or Mn1 in the S2 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 S2 → S3 process can be fit with two time constants, a very fast transition with a time constant of ~26 μs (τ1) followed by a slow one (τ2) with 317 μs (see green dashed line in Fig. 5C). Based on the kinetic H/D isotope effect, it was proposed that the fast phase of the S2 → S3 transition is connected to proton release to the bulk and the ~300-μs phase involves a proton-coupled electron transfer and oxidation of the Mn4Ca 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 OX from crystallography during the S2 → S3 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 S2 → S3 transition with better signal/noise level will be required.

**Water Dynamics during the S2 → S3 Transition.** In the current and recent (20) room-temperature crystal structures at 2.01 to 2.27 Å resolution, we observe ~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 CI1) 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 S2 state. When present in S1, 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 S2 → S1 transition. As W20 is already absent in the S2 state, it is not likely that W20 is the source of the substrate water that binds to the O5 position in the S2→S1 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 S0 → S1 transition but not in the S2 → S1 or S1 → S0 transitions (20, 63). W19, located in the vicinity of W20 in the dark state (S1), shows position changes between O4 and D1-S169. The shortest distance between W19 and O4 (2.4 Å) was observed in the S2 state (1F). The displacement of W19 between the 0F and 1F states appears as a negative electron density in the isomorphic 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 S2 → S1 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 OX appearance, a significant negative electron density is seen 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 of the O1 channel variability in position between the different illumination states, 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-Glu143) 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 OX Insertion. Water insertion during the S2 → S1 transition can be clearly followed by the buildup of the OX density between Ca and Mn1. However, the origin of OX, that is, how and where the water reaches the OX 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) WN1 (non-OEC ligated water near Mn1) → Mn1 (22), 2) W3 → Mn1 (28–30, 67), 3) W3 → Mn4 (14, 25, 28–30, 38, 68–71), or 4) WN2 (non-OEC ligated water near Mn4, for example W24 or W19) → Mn4 (36, 39, 72). In 1 and 2, water directly goes into the Mn1 open coordination site (OX) and the OEC remains in the open-cubane configuration throughout the S2 → S1 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 OX position in S1 [pivot/corousel mechanism (36, 39, 72)].

Among the above four proposals, the WN1 → 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 major 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 S3 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 OX site from W3 and refilled via the highly mobile waters in the O1 channel (case 2; see Fig. 7B).

Recently Suga et al. (21) reported the appearance of a new water next to residue CP43-Glu413 in their cryogenic 2F data in their crystallography and spectroscopy study correlates the structural changes occurring in the protein with the redox changes taking place at the metal center.

The earlier event we observe in this time-resolved study (Fig. 7A, Top) is the motion of YZ located between the primary donor, P680, 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 YZ (YZ → YZ+) to P680+ immediately after the charge separation between P680* (excited state of P680) and pheophytin. The oxidation of YZ is expected to change the H-bonding network along the residues D1-YZ-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 YZ. YZ is located about 4 Å away from Glu189 and the formation of the positive charge at the YZ/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. 7 A, 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 YZ triggers the shift of the Mn4 and Mn1 positions in the early stage of the S2 → S3 transition, which seems to be completed by ~150 μs.

Subsequent to the Mn1–Mn4 motion, OX becomes gradually visible in the OEC crystal structure.
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$_4$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$_4$Ca cluster are shown as a function of their distance from the Mn$_4$Ca cluster. The waters in the O1 channel are more mobile on average than the waters in the CI1 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.
visible in the electron density maps; it is still within the noise level at 50 μs, but it is at ~30% of its final occupancy by 150 μs, and ~80% by 250 μs, and close to full occupancy by 400 μs (Fig. 7B, Bottom). The oxidation of Mn1 appears to be directly coupled to the insertion of water, OX, 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 OX arrives as a ligand to Mn1 in the S3 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 (OX) insertion pathway that involves Mn4. Therefore, we hypothesize that OX 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 S2 → S3 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 Y2 and the proton transfer to the neighboring His190 (Y2 oxidation/His190+ formation), because it reduces the pKs of W3. Then, upon oxidation of the Mn cluster [five coordinate Mn1(III) → five coordinate Mn1(IV)] and reduction of Y2+, 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 (OX) 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 μs after the second flash (Fig. 5C and SI Appendix, Fig. S8) could suggest that Mn oxidation triggers OX insertion, although the data are also compatible with a faster structural rearrangement process that does not change the oxidation state of the MnCa cluster followed by concomitant Mn oxidation and OX insertion. Most theoretical studies have proposed a concerted mechanism that requires binding of water first before Mn oxidation. However, recently Chrysina

Fig. 7. Schematic of the S2 → S3 transition. (A) The sequence of events in time leading to the insertion of OX 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, YZ, 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 OX site and proton movements from W3 to Y2 via W25 or W4. The protonated oxo bridge at the OX position could form a hydrogen bond with the carboxylic chain of Glu189.
et al. (35), based on W-band EPR results, proposed oxidation of MnI prior to binding of water, which would also be consistent with our results presented above.

It has been proposed that an O5–O6 (or O6) peroxo-bond may form already in the S1 state (19). We clearly showed previously and confirm in the current study that no O5–O6 bond is formed in the S1 state or any of the time points during the S2–S3 transition. Moreover, our XES results do not show a reduction of Mn in the S1 state, that should accompany any formation of an O–O peroxo-bond. Instead, our data clearly show that O2 is a bridging ligand between Ca and MnI, and we propose that O2 is hydrogen-bonded to the carbonylate oxygen of Glu189 located at a distance of 2.4 Å (Fig. 7 B). Bottom. We note that O2 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 S1 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 S3 → S1 transition in PS II. In this transition the catalytic center is first oxidized one electron 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 O2, 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 closer to understanding the even more complex and highly or-

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