

Capturing the sequence of events during the water oxidation reaction in photosynthesis using XFELs

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Ever since the discovery that Mn was required for oxygen evolution in plants by Pirson in 1937 and the period-four oscillation in flash-induced oxygen evolution by Joliot and Kok in the 1970s, understanding of this process has advanced enormously using state-of-the-art methods. The most recent in this series of innovative techniques was the introduction of X-ray free-electron lasers (XFELs) a decade ago, which led to another quantum leap in the understanding in this field, by enabling operando X-ray structural and X-ray spectroscopy studies at room temperature. This review summarizes the current understanding of the structure of Photosystem II (PS II) and its catalytic centre, the Mn_4CaO_5 complex, in the intermediate S_i ($i = 0-4$)-states of the Kok cycle, obtained using XFELs.

Keywords: manganese metalloenzymes; oxygen evolving complex; photosystem II; water-oxidation/splitting; X-ray free-electron laser; X-ray spectroscopy

The water oxidation reaction in photosynthesis

The water oxidation reaction in nature is carried out by Photosystem II (PS II), a multisubunit membrane protein complex. This light-driven reaction is made possible by a series of spatially separated cofactors that extends over 40 Å, and includes the donor (the Mn_4CaO_5 catalytic centre), the reaction centre chlorophylls, and ends with the mobile quinone electron

acceptor (Q_B) [1,2]. Such chemical architecture provides an ideal platform to investigate the coupling of the one-electron charge separation reactions at the pico-second time scale with multi-electron/proton chemistry occurring at the millisecond time scale, using earth-abundant metal cofactors embedded in a flexible protein-water network. Understanding the insights

Abbreviations

DOT, drop-on-tape method; EPR, electron paramagnetic resonance spectroscopy; MIMS, membrane inlet mass spectrometry; XAS, X-ray absorption spectroscopy; XES, X-ray emission spectroscopy; XFEL, X-ray free-electron laser; XRD, X-ray diffraction.

from nature's design provides inspiration for how to build artificial photosynthetic devices, where the controlled accumulation of charge and high selectivity of products is currently challenging, especially if the catalysts are made of earth-abundant elements.

To solve the mechanism of the water oxidation reaction in PS II, our strategy has been to follow the structural and chemical sequence of events in the enzyme that lead to O–O bond formation, and to understand the cycle of the catalytic reaction at the atomic and electronic structure levels. The questions regarding this light-driven water oxidation reaction include: What structural parameters control reaction kinetics? How does the environment (protein and hydrogen-bonding network) change to accommodate a dynamically evolving energy landscape during the multielectron/multi-proton chemistry, while adjusting local pH and redox potential to drive chemistry? What are the features of PS II that activate the earth-abundant metals Mn and Ca, which are the building blocks of the catalytic centre of water oxidation in PS II, so that they can support highly efficient water oxidation catalysis? To answer these questions, the application of new methods in crystallography and X-ray spectroscopy at X-ray free-electron lasers (XFELs) plays a critical role.

X-ray free-electron lasers enable mechanistic studies of Photosystem II

The X-ray pulses generated by an XFEL are extremely intense with short pulse widths, containing as many photons (10^{12}) in one pulse (< 50 femtoseconds) as synchrotron beamlines provide in 1 s [3,4]. Moreover, the X-ray beam is very small in size, typically 2–5 μm or in some instances 100 nm in width, and therefore the sample/crystals being studied can be very small. These unique properties of the XFEL pulses make it possible to perform shot-by-shot X-ray data collection at room temperature: the femtosecond pulse is faster than the diffusion of radicals (picoseconds) caused by the X-rays in biological samples [5–7], and therefore the data can be collected before radiation-induced sample changes can develop, even at room temperature. This capability has made it possible to follow the structural and chemical changes in PS II in real time, as the reaction proceeds [8–11]. In PS II, this implies that one can access the reaction process at physiological temperature, and of particular interest is the S_3 to S_0 transition *via* the transient S_4 state; a step during which O–O bond formation, evolution of molecular oxygen, and substrate water binding occur. Unlike the stable intermediates (S_0 to S_3 states), the transient S_4 state and the transients between successive S-states

cannot be cryo-trapped, and therefore need to be studied at room temperature in a time-resolved manner.

To take full advantage of the capability of XFELs for the mechanistic studies of PS II, there have been several challenges that arise from the fact that at XFELs the sample is destroyed after each X-ray pulse. Establishing methods for preparing large volumes of suspension of highly diffracting microcrystals of defined size, delivering these samples efficiently at the pulse repetition rate of XFELs, *in situ* photoexcitation, and fast data analysis providing real-time feedback have been prime requirements [5,6,8–10]. While many different approaches using liquid jets [12–15] or rastering of solid targets [16,17] (see also [18–20] for general reviews on sample delivery) have been utilized so far to deliver PS II samples to the X-ray beam, each of them had shortcomings, for example, in terms of sample consumption, illumination precision, or available measurement modes. To circumvent these, we developed a robust drop-on-demand sample delivery setup for room temperature X-ray crystallography/X-ray emission spectroscopy (XES), with photochemical triggers to advance the S-states *in situ* (Fig. 1) [21]. Droplets that contain protein crystal suspension are formed by an acoustic droplet ejector that is connected to the sample well and that is automatically re-filled *via* a Hamilton syringe driven by a syringe pump. Typically, the droplets have a size of ~ 150 – $200 \mu\text{m}$ and contain several dozens of PS II single crystals (10 – $50 \mu\text{m}$ size). With this setup, we routinely achieve a droplet hit rate of close to 100% and a crystal hit rate of ~ 20 – 50% . Images with occasional multiple crystal hits can be deconvoluted and analysed separately. The setup allows us to take snapshots of crystallography and XES data with a pump-probe time delay between ~ 100 fs and up to several 10s of milliseconds and we can cycle PS II through its reaction cycle by exposing each single droplet to 1, 2, 3, or 4 light flashes before X-ray exposure. Using this setup [22,23] as well as other approaches [10,15], XFEL studies have started revealing the details of how water is oxidized by PS II and the role of the protein environment in the process. Below we discuss the interplay of structural changes of the overall protein and the chemical changes occurring at the metal site as PS II cycles through the enzymatic reaction.

Figure 2 shows the stepwise changes of the electron density at the oxygen-evolving complex (OEC) during the oxygen-evolving cycle, which are captured using the setup shown in Fig. 1. The electron density map ($2mF_{\text{obs}} - DF_{\text{calc}}$, green to blue) and O5/Ox omit map density (pink to red) of the 0F–3F states (measured 200 ms after illumination of the sample by 0, 1,

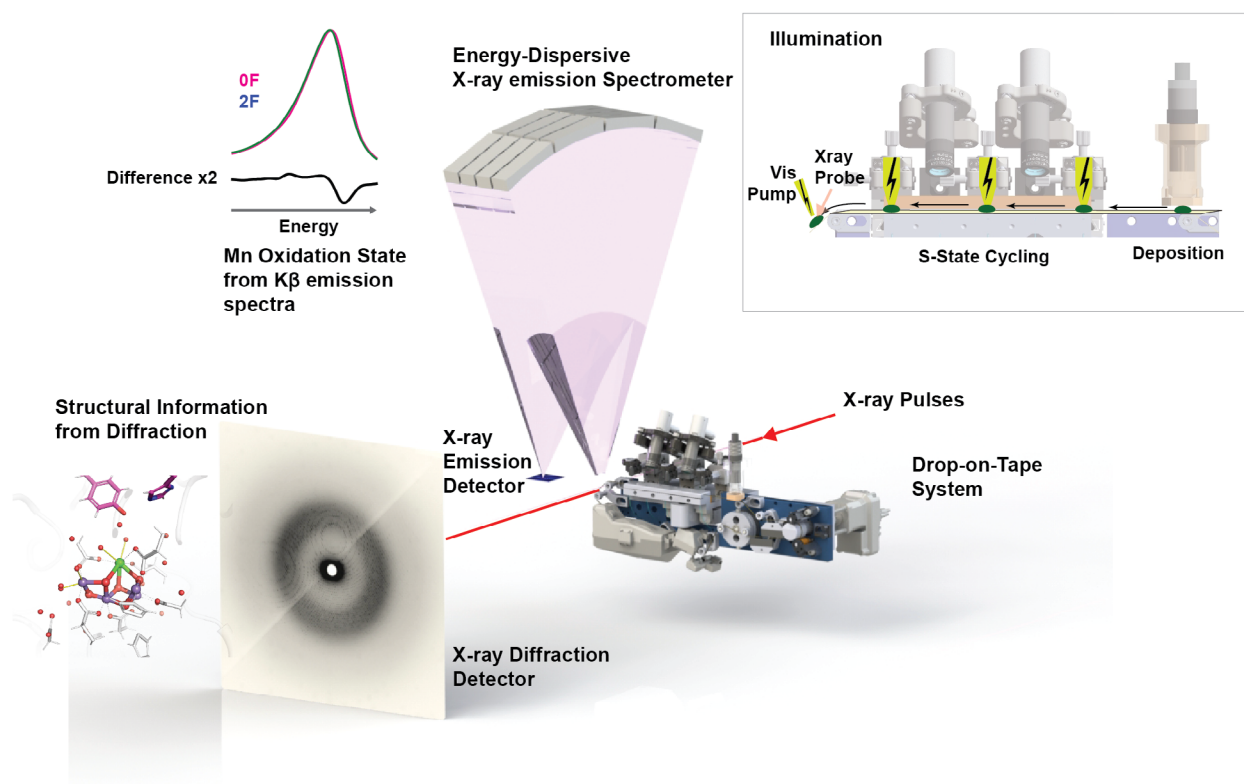


Fig. 1. Setup for delivering protein crystal suspension to the X-ray intersection point to perform simultaneous X-ray crystallography and X-ray emission spectroscopy. The Drop-on-Tape system (centre) [21] uses acoustic pulses to generate nl-sized droplets of protein crystal suspensions which are deposited on a polyimide transport tape. The droplets are then illuminated by up to three laser flashes before being transported into the X-ray interaction region (top right). Here, a 4th laser pulse can be used for time resolved optical pump-X-ray probe measurements. The forward scattering is recorded for crystallographic analysis (bottom left) while the X-ray emission signal (top left) is measured utilizing an energy dispersive spectrometer mounted above and a detector located sideways of the interaction point.

2, or 3 light flashes, respectively) are overlaid. The 0F and 1F data have a dominant population (> 90%) of the S_1 and S_2 states, respectively, and therefore they are considered as containing only a single species. On the other hand, 2F and 3F data are more mixed due to PS II's intrinsic miss parameters, which lead to a desynchronization of the states [9,22,24]. The S-state population of these flash states is estimated *via ex situ* methods of the same sample (electron paramagnetic resonance spectroscopy (EPR) and membrane inlet mass spectrometry (MIMS)) [25,26] as well as an *in situ* method (XES), which is carried out simultaneously with X-ray crystallography [27]. Based on the population analysis, we extracted pure S-state structures from the flashed data, which are shown along with the electron density map of the flashed states in Fig. 2. The likely assignment of Mn oxidation states (Mn^{3+} is depicted in orange, Mn^{4+} in purple) as well as protonation and deprotonation reactions are indicated for each S state. In the S_1 state, the cluster is in

a distinct 'right-open' structure with no bond between Mn1 and O5; the Mn4–O5 distance is about 2.2 Å, whereas the Mn1–O5 distance is about 2.7 Å, with Mn1 in a pentacoordinate ligand environment. Upon the transition from S_1 to S_2 , the structure of the cluster remains fundamentally unchanged, but one Mn, likely Mn4, is oxidized from +3 to +4. The S_3 state is unique as Mn1 has gained an additional, sixth oxygen ligand that forms a bridge between Mn1 and Ca.

To understand the sequence of events leading to the insertion of this new oxygen bridge, we recorded diffraction and Mn $K\beta$ XES data at several time points during the $S_2 \rightarrow S_3$ transition. We observed that the tyrosine Y_Z residue, located between the reaction centre chlorophylls and the OEC, shows some movement by 50 μs after the 2nd flash, along with the His190 residue that is hydrogen-bonded to Y_Z . Then, the Glu189 side chain, which bridges Mn1 and Ca and is located close to His190, moves away from Ca. This is followed by Mn1 and Mn4 moving apart as the

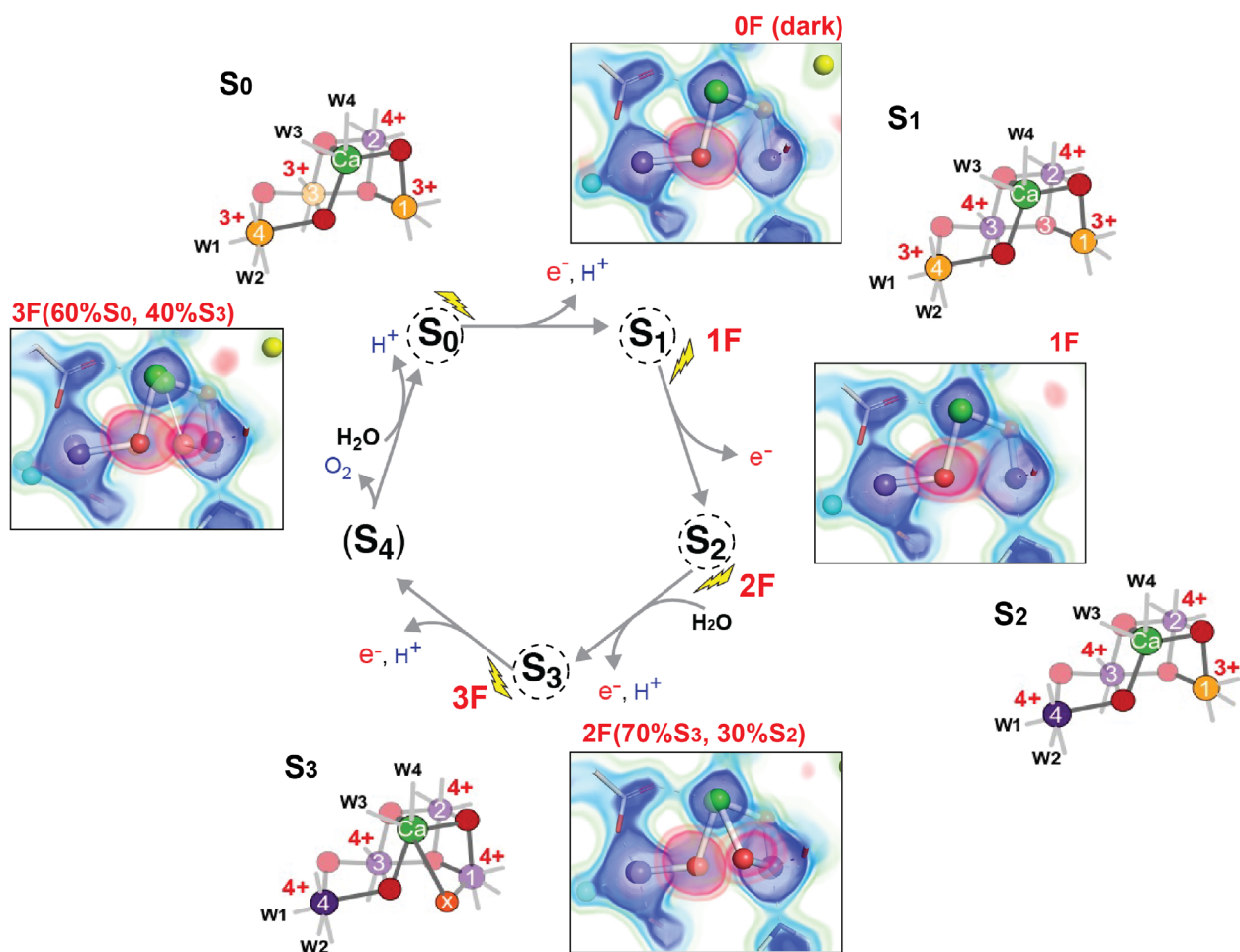


Fig. 2. Kok cycle of the water oxidation reaction. The reaction cycle of light-driven water oxidation in PS II is shown in the centre. Starting in the dark stable S₁ state each light flash given to the system advances the Mn₄CaO_n cluster by one oxidation state with S₃ being the highest oxidized stable intermediate state. The next photon triggers the formation and release of O₂ via the transient S₄ state and relaxation of the cluster to the most reduced S₀ state which returns to the S₁ state by another light flash. Electron density (2mF_{obs} – DF_{calc}) at different contour levels (1.5, 3 and 4 σ, green to blue) and omit electron density (mF_{obs} – DF_{calc}, contoured at 3 (orange) and 4.5 σ (red)) for the O₅ and O_x oxygen atoms are shown together with structural models of the OEC in each of the S-states. Mn are shown as orange (oxidation state 3+) or magenta (oxidation state 4+) spheres, O shown as red spheres and Ca as green sphere. Metal ligation by the protein is indicated by grey lines, except for Ca, where protein ligands are omitted for clarity. Adapted from [9].

extra oxygen bridge (O_x(H)) is inserted at the open coordination site of Mn1 and Ca. Our structural data indicate that O_x could be supplied via a ‘water wheel’ like arrangement of five waters next to the OEC that is connected by the ‘O1 channel’ (Fig. 3) to the bulk solvent. We hypothesize that the Ca coordination environment, for example, W4 → W3 → O_x, is used as a path for the water insertion to Mn1. XES spectra show that Mn oxidation (τ of ~ 350 μs) during the S₂ → S₃ transition mirrors the appearance of O_x electron density, showing that insertion happens in parallel to Mn1 oxidation from Mn³⁺ to Mn⁴⁺. Thus, in S₃ all four Mn seem to be oxidized to a formal oxidation state of 4+ [8].

Upon the 3rd flash, the S₃ state advances to the S₀ state, which shows the original right-open configuration of the cluster (Fig. 2), but with one of the Mn–Mn distances (Mn3–Mn4) slightly elongated due to the presence of Mn³⁺ and the protonation of one μ-oxo bridge. During this reaction step, O₂ is formed and released, and one additional water needs to bind to restore the Mn₄CaO₅ cluster. In addition, two protons are released, one before O–O formation and one concomitant with O₂ release and water binding [1,22,28,29]. These reaction steps are presently being studied by XFEL.

The ability to advance the reaction through the Kok cycle *in situ*, evidenced by the crystallography and

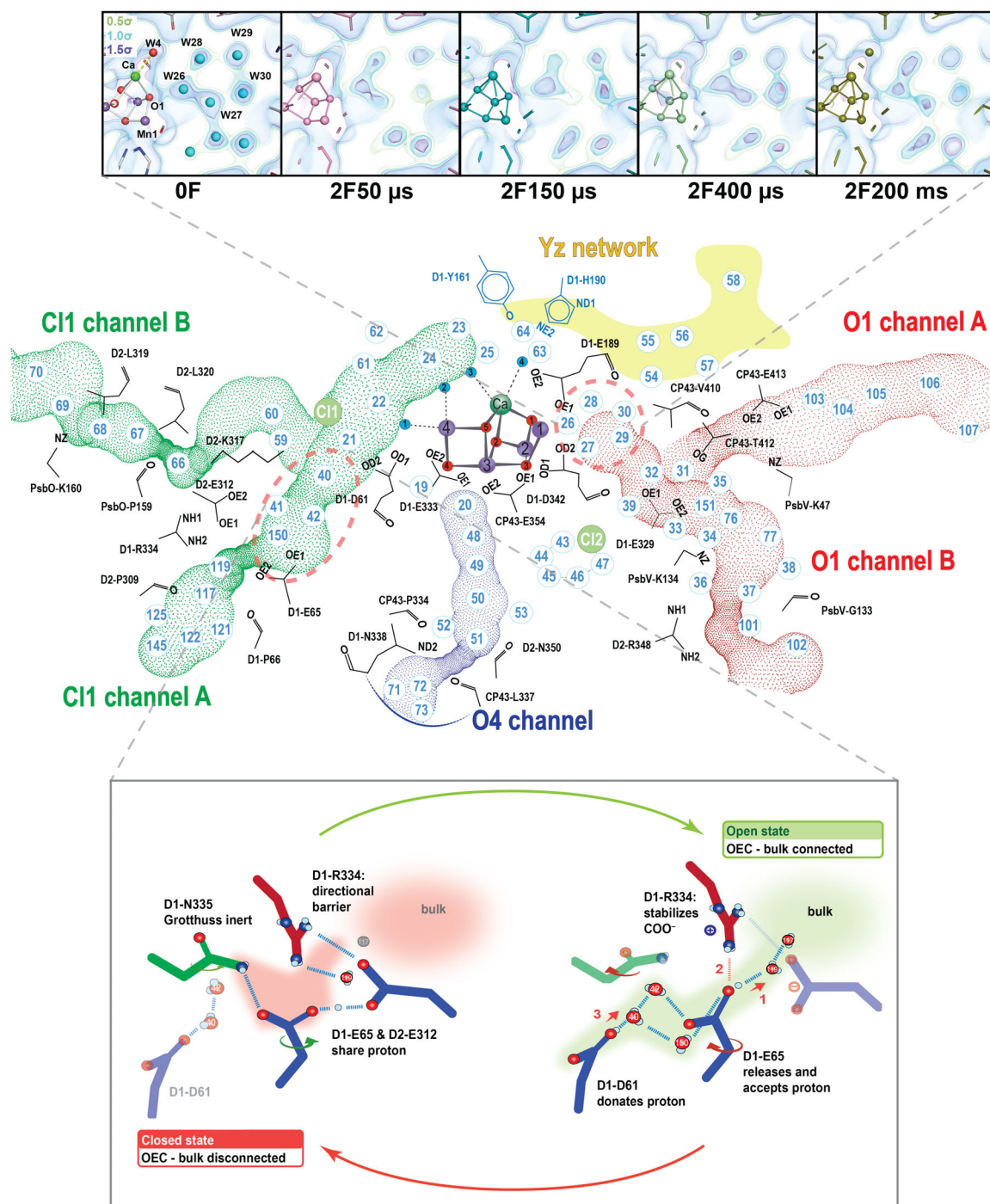


Fig. 3. The environment of the Mn cluster in PSII. The Mn₄CaO₅ cluster (Mn shown as blue, Ca as green, oxygen as red spheres) is embedded in a network of water channels (centre, adapted from [9]) connecting it to the bulk. The main channels are labelled ('O1 channel', 'O4 channel', and 'C1 channel'). Waters located in the room temperature X-ray structures are indicated by numbered circles and selected residues lining the channels are labelled. A pentamer of waters (W26-W30) located next to the O1 atom of the Mn cluster is indicated by a dashed circle. Changes in the electron density of this 'water wheel' region at different time points in the S₂ to S₃ transition are clearly visible (top, adapted from [8]). These indicate high water mobility and a possible role of the water wheel and the O1 channel in water transport to the Mn cluster related to the water insertion event that takes place upon formation of the S₃ state. A bottleneck in the C1 channel (highlighted by a dashed oval) changes configuration at around 150 μs into the S₂ to S₃ transition (bottom, adapted from [39]). This opening and closing of a potential 'proton gate' could facilitate proton transfer from the cluster to the bulk during a specific time window in the S₂ to S₃ transition.

X-ray spectroscopy results at XFELs [8,9], provides evidence that PS II reaction centres are fully active in the crystalline environment under the physiological temperature that is used during the experiments (Fig. 1). In addition, it demonstrates that our efficient drop-on-demand sample delivery to the X-ray intersection points (Fig. 1), which avoids dehydrating crystals, efficiently prevents the loss of enzyme activity, decrease of diffractivity, and the release of Mn^{2+} . There has been a debate about whether multiple OEC configurations exist in each S-state due to spin isomerization and/or different protonation states of oxygens [30–32]. Determining positions of heavy atoms like Mn is highly accurate at the current resolution of 2.0 Å, and no clear evidence of multiple metal positions were observed in each S-state. On the other hand, the expected structural differences between multiple oxygen positions due to different protonation states are more subtle and require higher resolution diffraction data. We, however, note that within our lower detection limit of ~10% of the population, the S-state models shown in Fig. 1 are the dominant configuration, and the minority fractions, if they exist, will not be accurately detected at the current crystallography resolution of ~2.0 Å.

Besides the function of the catalytic centre, the importance of the protein environment, including the hydrogen bonding network, has been recognized more and more in the field of bioinorganic catalysis. In PS II, water and proton pathways are required to facilitate substrate delivery to and proton egress from the catalytic site. Such channels have been proposed from theoretical studies built on the crystallography data [33–38]. Out of the three main channels – ‘O1 channel’, ‘C11 channel’, and ‘O4 channel’ – identified in the room temperature structures (Fig. 3), recent structural studies propose that the O1 channel is the likely water intake pathway [8,39]. This hypothesis is based on the high mobility of waters located along the O1 channel. On the other hand, the C11 channel is the likely proton release pathway in this transition, based on the structural rearrangements of water molecules and amino acid side chains along these channels [8,39]. The reversible rotation of the amino acid residue D1-E65 is observed in the time resolved room temperature data (Fig. 3 bottom), and it is hypothesized that this area serves as a gate for proton transport that plays a role in minimizing the back reaction [39]. The results indicate that the water oxidation reaction at the OEC is well coordinated with the amino acid side chains and the hydrogen-bonding network over the entire length of the channels, which is essential in shuttling substrate waters and protons. A similar study is

necessary for the other S-state transitions, in particular, for the S_3 to S_0 transition where the catalytic centre resets the process by four electron reduction of the Mn_4CaO_6 cluster, along with O_2 release.

Conclusions and perspectives

The progress of the room temperature structural insights into PS II and its catalytic mechanism summarized here is based on more than a decade-long development of the XFEL-based X-ray methods, and more specifically the development of sample preparation [40] and sample introduction methods into the X-ray pulses with multiple-illumination protocols required for PS II samples [21], and constant improvements on analysis methods for serial crystallography data from XFELs [41–43]. The first decade of XFEL science has brought us many striking results and the emergence of exciting new research fields in the biosciences area. However, much work still lies ahead when it comes to making the best use of this powerful tool. Similar concepts have been applied to studies of other metalloenzyme reactions that are triggered by mixing with a gas or by chemical mixing with a substrate [21,44,45]. Understanding the function of metalloenzymes, which are very important in the field of human health and of relevance to renewable energy, will greatly benefit from current and future XFEL and the X-ray methods described that make it possible to follow the atomic and electronic structural changes, in real time, at room temperature, as the metalloenzymes progress through the catalytic reactions.

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