Taking snapshots of photosynthetic water oxidation using femtosecond X-ray diffraction and spectroscopy

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The dioxygen we breathe is formed by light-induced oxidation of water in photosystem II. O2 formation takes place at a catalytic manganese cluster within milliseconds after the photosystem II reaction centre is excited by three single-turnover flashes. Here we present combined X-ray emission spectra and diffraction data of 2-flash (2F) and 3-flash (3F) photosystem II samples, and of a transient 3F’ state (250 ms after the third flash), collected under functional conditions using an X-ray free electron laser. The spectra show that the initial O–O bond formation, coupled to Mn reduction, does not yet occur within 250 μs after the third flash. Diffraction data of all states studied exhibit an anomalous scattering signal from Mn but show no significant structural changes at the present resolution of 4.5 Å. This study represents the initial frames in a molecular movie of the structural changes during the catalytic reaction in photosystem II.
Aerobic life on earth is supported by the constant regeneration of dioxygen through photosynthetic water oxidation in green plants, algae and cyanobacteria. This reaction takes place in photosystem II (PS II), a multi-subunit membrane protein complex. PS II couples the one-electron photochemistry of the primary charge separation at the reaction center with the four-electron redox chemistry of water oxidation at the Mn₄O₄Ca cluster of the oxygen evolving complex (OEC) at the luminal side of the protein complex, using the spatial and temporal organization of the electron donor and acceptor cofactors in PS II₁–⁵. This well-controlled electron and proton flow results in the high quantum efficiency of PS II.

During the water oxidation reaction, the OEC functions as a redox capacitor by storing four oxidizing equivalents before the release of molecular oxygen. Starting from the dark stable S₀ state, the oxidation state of the OEC is increased by one on each light excitation of PS II until the highest oxidized stable intermediate state, S₅, is reached. Following the next light excitation, the OEC is oxidized one more time to form the transient S₁Y²ₓ and S₄ states that lead to dioxygen formation, which converts the OEC to its most reduced state, S₀ (ref. 3). The fourth light excitation sets the OEC back to the S₁ state, and thereby completes the cycle (Fig. 1a).

Much structural and mechanistic information about PS II, the OEC and the O-O bond formation was gained through mass spectrometrical, various spectroscopic⁴⁻⁹, crystallographic¹⁰⁻¹³ and theoretical¹⁴⁻¹⁶ studies over the past decade. In particular, the most recent structure, inferred from X-ray diffraction (XRD) data, has provided detailed geometric information of the OEC, including ligands and bound water molecules.¹² Most of the experimental studies, however, are carried out at cryogenic temperatures and represent a static picture of the system in a frozen state. Although the stable intermediate states, S₅ through S₀, can be trapped and studied at cryogenic temperatures, the critical S₅ → S₁Y²ₓ → S₁ → S₀ step—where dioxygen is formed, two protons and O₂ are released, and where at least one substrate water binds—only occurs under ambient conditions and has no intermediates that can be cryotrapped. To date, there has only been one transient X-ray spectroscopy study of the S₁ → S₁Y²ₓ → S₁ → S₀ transition performed at room temperature (RT).¹⁷ More detailed investigations of the transient states by X-ray spectroscopy and by X-ray crystallography have been hampered due to the severe radiation damage, especially to the Mn₄CaO₄ cluster that is significantly faster at RT as compared with cryogenic conditions. However, X-ray-induced changes, particularly at the redox-active metal site, have even been an issue for experiments carried out at cryogenic temperatures.¹⁸⁻²⁰.

We have recently introduced a combined spectroscopy and diffraction data collection methodology at RT¹¹ using the ‘probe before destroy’ method¹²⁻²₅ made possible by the ultra-short (fs) and bright X-ray pulses of an X-ray free electron laser (XFEL). In this approach, XRD data and Mn Kβ-X-ray emission spectra (XES), sensitive to the metal charge density²⁵,²⁶, are measured simultaneously from micrometre-sized crystals of PS II, thereby obtaining information about the geometric and the electronic structure of the active site, under identical conditions. Owing to the ultra-short fs X-ray pulse duration, the sample is probed before the manifestation of X-ray induced changes—which predominantly take place on the picosecond time scale (for damage to the atomic structure)—even under ambient conditions. One should note that with conventional synchrotron X-ray sources the main source of radiation damage is via the generation of radicals from the solvent (water). Subsequent diffusion of these radicals leads to specific damage (for example, reduction of metal sites) and modification of amino acid side chains (for example, decarboxylations). Such events are diffusion controlled and occur on a longer time scale (>picoseconds) and seem not to be dependent on the dose rate. Earlier work¹¹,¹² showed that the approach of using ultrafast (<50 fs) and ultra-bright (10¹² photons per pulse) X-ray pulses permits the collection of XES and XRD data from intact PS II, and we reported results from the dark-adapted (S₁) and the one flash (S₃) samples with an XRD resolution limited to 5.5 Å. Here we present XES and XRD data from the last step of the Kok cycle, where O₂ is evolved, with an improved resolution of 4.5 Å. This step, triggered by the third flash given to dark-adapted PS II samples, advances the PS II complex from the S₁ to the S₃ state, via the transient S₁Y²ₓ and S₄ states (S₅ → S₁Y²ₓ → S₁ → S₃ transition). Furthermore, we observe an anomalous signal for the Mn atoms in the OEC from all the states, including the transient S₁Y²ₓ state. This observation supports the quality of our XRD data and also the data analysis protocols, and we envision that the Mn anomalous signal could be used as a sensitive probe for monitoring changes of the atomic positions of Mn in the OEC during the catalytic cycle in future studies at higher resolution.

Results

XES at different time points in the catalytic cycle. PS II was advanced through its reaction cycle in situ, using a flow/illumination scheme (Fig. 1b) employing an electrospun liquid jet. The protocol consisted of visible-laser illumination through three optical fibres directly attached to the sample delivery capillary, and an additional laser for illumination of the sample in the jet.

Figure 1 | Flash-induced changes in PS II and experimental set-up used at LCLS. (a) Kok cycle describing the different stable intermediate states of the catalytic water oxidation reaction in PS II. (b) Scheme for the illumination set-up used to advance PS II in the catalytic cycle and measure simultaneously the XRD and XES signal at LCLS. Lasers 2 and 3 were used to generate 2F samples, lasers 1, 2 and 3 for 3F samples and lasers 2, 3 and 4 to generate the 3F₀ samples.
The temporal frequency for illumination was chosen to match the sample flow rate, so that each volume segment was illuminated by each fibre once while passing through the capillary. The set-up also allows enough time (~0.5 s) for complete PS II turnover between consecutive illuminations, which takes into account the slower acceptor side reactions7,29-31, while being rapid enough to avoid significant decay of the S-states, that are stable for on the order of several tenths of seconds. The fourth laser (labelled 'laser 4' in Fig. 1b) illuminated the sample in the jet, to study transients during the S3 to S0 transition by changing the timing between the third visible-laser pulse and the X-ray probe pulse.

O2 detection via membrane-inlet mass spectrometry (MIMS) was used for optimizing the conditions for S-state turnover in the capillary flow sample delivery system, using a facsimile of the flow/illumination set-up employed at Linac Coherent Light Source (LCLS) (see Methods). One of the most important factors in the illumination scheme is the required light intensity for efficient turnover through the S0 state cycle. Too low intensities can lead to only partial turnover of the samples, while too high intensities increase the miss parameter via light scattering along the capillary, and may also inactivate the sample. The optimal light intensity can be found by the quality of the O2 oscillation pattern, and also by the total O2 produced per PSII complex and flash number. The former method should normally be sufficient, but a small uncertainty remains whether there can be a certain part of the sample that never sees any light, and thus does not contribute to the oscillation pattern. To address this question, the latter method needs to be employed (see Methods), which requires the absolute calibration of the MIMS signals. The amount of 0.73 O2/RC after three flashes (Fig. 2a) shows that the light conditions used for illumination are optimal for saturating all PSII reaction centres in the sample. The O2 evolution patterns obtained from PS II solutions and PS II microcrystals (Fig. 2b) show light-induced turnover of the catalytic cycle as expected. Analysis of the flash pattern indicates that the S3 state is the majority component (≥55%) in the samples given two visible-laser flashes (2F) with virtually no S0 state present. In contrast, the largest component in the 3F samples is the S0 state (≥40%). Therefore, the difference between the 3F and 2F samples is dominated by the formation of the S0 state at the expense of the S1 state.

We measured XES on PS II solutions at the Coherent X-ray Imaging (CXI) instrument18 at LCLS (see Methods). As shown in Fig. 3, a clear shift between the 2F (S1-enriched) and 3F (S0-enriched) spectra is observable. Calculation of the first moment (see Methods) revealed that the 3F spectrum is shifted ~0.1 eV to higher energies indicating a reduction of Mn26, as expected for the transition of the OEC from the highly oxidized S1 to the most reduced S0 state, in which the formal oxidation states are assigned as MnI2 and MnIIIIV, respectively1,2,6,33,34. Comparing these data to synchrotron radiation (SR) data collected at cryogenic temperature from Thermosynechococcus elongatus PS II and previously recorded data from spinach PS II35 shows a very similar trend (Fig. 3c and Supplementary Fig. 1).

In addition to the 2F and 3F spectra, we measured the XES at a time point 250 μs after the third flash (3F′) using lasers 2, 3 and 4 (Fig. 1). The XES for this transient state is similar in position to the 2F spectrum (Fig. 3a,b), but its shape is different with broadening towards the lower energy side. Although such broadening could be caused by oxidation of a fraction of the lower S-states (S1 and S2) in our sample, it could also be due to light-induced changes in the electronic structure of the S3 fraction. Nevertheless, the result shows that there is no significant reduction or oxidation of the Mn taking place within the 250-μs time span between the third visible-laser excitation pulse and the X-ray probe pulse.

Figure 2 | Oxygen production by PSII. (a) O2 yield per PSII reaction centre as detected by MIMS as a function of flash number (measurement shown is for PS II solutions, flow rate 0.5 μl min−1, frequency 4 Hz, light intensity was 7 μl for each fibre). (b) O2 yield measured by MIMS as a function of flash number from PS II solutions (black) and PSII microcrystals (red).

XRD in the higher S-states. XRD data from 2F (S1-enriched), 3F (S0-enriched) and 3F′ (S1Y0.5Z0.5-enriched; 250 μs after the third flash) PS II crystals, as well as in the dark state (S0), were collected. Microcrystals of PS II were prepared using a new seeding protocol (see Methods). Clear Bragg spots were observed to a resolution of ~4.1 Å, with thermal diffuse scattering extending well beyond this to ~3.0 Å, indicative of correlated atomic motion in the crystal. For the 2F data, a total of 16,973 indexed patterns were merged resulting in a data set of 4.5 Å resolution (see Table 1 and Supplementary Tables 1 and 2 for details). The resolution cutoff for the merged data sets was chosen based on the resolution dependence both of the multiplicity and of CC1/2, the correlation coefficient of semi-data sets merged from odd- and even-numbered images36; that is, completeness >90%, multiplicity >6 and CC1/2 >30%. Likewise, data sets of 3F, 3F′ and 0F states were obtained with resolutions of 4.6 Å (13,094 lattices), 5.2 Å (7,850 lattices) and 4.9 Å (6,695 lattices), respectively (Table 1 and Supplementary Tables 1 and 3–5). Electron density maps for all four states are shown in Fig. 4 and Supplementary Figs 2 and 3. A comparison with the SR data cut to the same resolution shows that the level of detail visible is as expected for this resolution range (Supplementary Fig. 4). The occupancy for selected non-protein molecules was set to zero and the simulated annealing omit maps were computed for all data sets, to remove potential model bias arising from phasing with a complete, high-resolution starting model (pdb: 3bz1)37. The
result clearly shows the electron density of the $\text{Mn}_n\text{O}_x\text{Ca}$ cluster, the non-haem Fe, the chlorophyll and even partially for the quinone cofactors (Supplementary Fig. 3) in the $mF_o - DF_c$ difference maps. The regions around the OEC, the acceptor-side quinones and non-haem iron, where the largest changes are expected, were inspected for changes between the different states. No statistically significant changes were observed in the $2mF_o - DF_c$ maps of the individual data sets (Fig. 4a,b and Supplementary Figs 2 and 3) and in the isomorphous difference maps ($mF_o - mF_c$) between the different data sets (Fig. 4c,d and Supplementary Fig. 5). This shows that any structural changes related to the $S$-state transitions are smaller than what we can detect at the current resolution. However, it should be noted that the $mF_o - DF_c$ Fourier maps contain several features that are observed consistently in both monomers and all flash states; namely, an electron density peak at the position of the OEC when viewed at a contour level of $+3\sigma$, a small peak 10 Å distant that appears to be coordinated by residues Glu 333 and Asp 61 of the D1 polypeptide, and other nearby peaks. Smaller negative peaks are seen at the $-3\sigma$ contour, for example, close to Val 185 and Phe 182 of the D1 protein (Supplementary Fig. 6).

**Measurement of anomalous XRD signal from Mn in PS II.** Accurate determination of the Bragg spot intensities and the derived structure factors is challenging for single-shot crystallography at XFELs\textsuperscript{21,23,24}. As a control to validate the data quality and our analysis protocol, we investigated whether small anomalous differences could be detected in the recorded Bragg spot intensities. Such differences between inversion-related Bragg spots (Bijvoet pairs) arise from the collection of diffraction data at energies above an absorption edge and are often only in the order of $\sim 1\%$ of the total signal intensity. We used an incident energy of 7.1 keV in our current XES/XRD data collection, which is close to the Mn edge (6.54 keV), and favours observing the anomalous signal from Mn in the OEC.

As a positive control of the methodology, we first analysed microcrystal diffraction data from a model system, thermolysin, which natively binds one Zn and several Ca ions\textsuperscript{27}. Data from thermolysin microcrystals were collected at 1.27 Å (9.76 keV), $\sim 100$ eV above the Zn edge (9.66 keV). Diffraction was observed out to the corners of the detector (1.50 Å) and the integrated intensities were merged to obtain a data set to 1.80 Å resolution (Table 1 and Supplementary Table 6). Analysis of the Bijvoet pairs in the merged data showed a clear anomalous signal contribution, and anomalous difference maps showed a clear maximum, $18\sigma$ above the mean, located at the position of the Zn ion as well as lower maxima for three of the four Ca ions and for the sulphur of one of the methionine residues (Fig. 5a,b and Supplementary Fig. 7).

In PS II, a clear anomalous signal (Fig. 5c,d, Fig. 6 and Supplementary Figs 8 and 9) from Mn in the OEC is also detected in all four data sets (0F, 2F, 3F and 3F$'$) (Supplementary Table S1). Figure 5c,d shows the anomalous difference map from the 3F data after omitting the OEC and performing simulated annealing refinement. It is evident from the overview shown in Fig. 5c that the largest peak ($\sigma = 6$) in the anomalous density is located at the position of the OEC. The density covers the Mn ions in the cluster and does not include the Ca (Fig. 5d) as expected from the weaker anomalous contribution of Ca at 7.1 keV ($f''$ of 1.6 for Ca compared with 3.4 for Mn at 7.1 keV). Similar results were obtained for the other PS II data sets for both monomers in the PS II dimer (Fig. 6 and Supplementary Figs 8 and 9). It should be noted however that the anomalous difference Patterson maps did not reveal peaks above the noise level attributable to Mn. This result is expected as also the anomalous data measured at SR sources at 3.5 Å resolution\textsuperscript{10} did not yield any peaks in the Patterson map above the noise level, due to the large protein mass and the low number of anomalous scatterers per unit cell volume.

**Discussion**

The quality of the PS II XRD data reported here for the $S_1$ state is improved compared with the previously obtained XFEL data: 4.5 Å versus 5.7 Å\textsuperscript{21}. Owing to the inherent fluctuations in pulse intensity, crystal size and crystal quality in single-shot microcrystal experiments at an XFEL, the signal strength varies...
from shot to shot. Therefore, we expect a distribution of diffraction images with different maximum resolution. To avoid adding noise into the diffraction data, an individual resolution cutoff was computed for each diffraction image based on the signal strength (see Methods). The observed distribution of the resolution for PS II as well as for thermolysin explains why the multiplicity in both cases (Supplementary Tables 2–6) decreases steadily in the higher resolution shells.

Recently, the first observation of an anomalous signal from femtosecond diffraction experiments with microcrystals at an XFEL38 and the first successful de novo phasing of lysozyme at 2.1 Å resolution using the anomalous signal of gadolinium Table 1 | Statistics for processed data and refined structures.

|                      | Dark (S1) | 2-flash (2F) | 3-flash + 250 µs (3F') | 3-flash + 500 ms (3F) | Thermolysin |
|----------------------|-----------|--------------|------------------------|-----------------------|-------------|
| Wavelength (Å)       | 1.77      | 2.07         | 2.07                   | 2.07                  | 1.27        |
| Resolution range (Å) | 72.93–4.9 (5.08–4.9) | 72.97–4.5 (4.66–4.5) | 68.41–5.2 (5.39–5.2) | 72.96–4.6 (4.76–4.6) | 34.27–1.80 (1.86–1.80) |
| Space group          | P 2 1 2 1 | P 2 1 2 1   | P 2 1 2 1              | P 2 1 2 1            | P 6 2 2     |
| Unit cell dimensions (Å) | 132.9     | 132.9        | 132.6                  | 132.4                | 93.0        |
|                      | 229.0     | 228.7        | 229.3                  | 228.8                | 93.0        |
|                      | 307.7     | 308.0        | 306.8                  | 307.9                | 130.4       |
| Unique reflections   | 41,292 (4,013) | 52,965 (5,008) | 34,679 (3,378)        | 49,771 (4,812)       | 31,458 (3,075) |
| Completeness (%)     | 99.7 (98.6) | 99.5 (95.8) | 99.7 (98.1)            | 99.7 (98.2)         | 100.0 (100.0) |
| Wilson B-factor      | 172       | 172          | 176                    | 176                  | 16.4        |
| R-work               | 0.281 (0.363) | 0.276 (0.367) | 0.271 (0.347)         | 0.278 (0.371)       | 0.208 (0.349) |
| R-free               | 0.292 (0.337) | 0.284 (0.393) | 0.289 (0.378)         | 0.284 (0.346)       | 0.232 (0.368) |
| Number of non-hydrogen atoms | 50,244     | 41,052      | 9,192                  | 0                    | 2,740       |
| Macromolecules       | 2,740     | 2,415        | 5                      | 324                  | 2,415       |
| Ligands              | 100.0 (100.0) | 100.0 (100.0) | 100.0 (100.0)         | 100.0 (100.0)       | 100.0 (100.0) |
| Wilson B-factor      | 0.005     | 0.005        | 0.005                  | 0.005                | 0.005       |
| RMS (bonds) (Å)      | 0.005     | 0.005        | 0.005                  | 0.005                | 0.005       |
| RMS (angles) (°)     | 0.75      | 0.75         | 0.77                   | 0.75                 | 0.92        |
| Ramachandran favoured (%) | 91        | 91           | 91                     | 91                   | 95          |
| Ramachandran outliers (%) | 1.2       | 1.2          | 1.1                    | 1.2                  | 0           |
| Clashscore           | 9.43      | 9.45         | 9.50                   | 9.34                 | 1.72        |
| Average B-factor (Å²) | 207       | 174          | 208                    | 180                  | 19.6        |

Statistics for the highest-resolution shell are shown in parentheses. All unit cell angles are 90° for photosystem II structures, and a = b = 90°, c = 120° for thermolysin.

**Figure 4 | Electron density maps obtained for PS II.** (a) 2mFo – DFc maps for the dark and (b) the 2F data of PS II are shown in grey contoured at 1.0σ, mFo – DFc maps after omitting the OEC are shown in green and red, contoured at ± 5.0σ. (c) mFo – mFo isomorphous difference maps for the 2F–dark data and (d) the 3F–2F data are shown for both monomers and are contoured at ± 3σ (bright green, monomer I; pale green, monomer II) and ± 3σ (red, monomer I; salmon, monomer II) together with the model for the 2F data (Mn shown as magenta spheres, Ca as white sphere).
**Figure 5 | Anomalous signal in the XFEL data sets.** (a) Anomalous difference map of the thermolysin data after simulated annealing with the occupancy for Zn and Ca set to zero to minimize model bias. The map is contoured at 4.0σ, extending over the entire thermolysin molecule. The position of the highest peak in the map (Zn atom) is highlighted. (b) The same anomalous difference map of thermolysin shown in the region of the natively bound Zn ion (magenta sphere), contour level at 3.0σ. (c) Anomalous difference map obtained from the 3F data of PS II, shown for one monomer, location of the strongest peak is highlighted, contour level at 4.0σ. (d) Enlarged view of the 3F anomalous density for the region of the OEC (contoured at 4.0σ; Mn shown as magenta spheres, Ca as white sphere). All maps shown are anomalous difference simulated annealing omit maps.

**Figure 6 | Anomalous signal from Mn for different illumination states of PS II.** (a) Anomalous map of the OEC in PS II is shown for the 2F data (magenta) in monomer I. (b) Anomalous map of the 3F data in monomer I. (c) Anomalous map of the 2F (cyan) and 3F (magenta) data in monomer I, orientation is rotated by 90° around horizontal and vertical axis compared with the view in a. (d) Anomalous map for monomer II, 2F (cyan) and 3F (magenta) data are shown, view direction is similar to c. All maps shown are anomalous difference simulated annealing omit maps contoured at 3σ.
obtained in an XFEL experiment were reported. In the gadolinium phasing experiment of lysozyme, the anomalous signal strength was around 5–15%. In comparison, we expect an anomalous signal of <1% for Mn in PS II and of ~1.5% for Zn in thermolysin. The observation of the very strong anomalous peak for Zn in the thermolysin data indicates that the Bragg peak intensities were determined with sufficient accuracy to extract the weak anomalous difference (see ref. 40 for a report on determining the anomalous Zn signal of thermolysin from SR measurements). Furthermore, the presence of the anomalous density for Mn in all of the PS II data sets, despite the expected low signal strength, confirms the quality of the data and implies that structure factors can be extracted reliably from the current PS II data sets. In this regard, it should be noted that even the high-resolution shell of the data still contains a considerable amount of anomalous signal as can be seen in Supplementary Fig. 9B.

As described above, the electron density of PS II shows the level of detail expected at the specified resolutions (4.5–5.2 Å, depending on number of collected images per S-state; Supplementary Fig. 4). The quinone co-factors (Q states in the dark, 2F and 3F data; Qh in the 3F data), that were not visible in the previous XFEL data due to limited resolution, are now partially visible in the mF0 − DF0 difference maps (Supplementary Fig. 3). In the earlier SR XRD structures of PS II with a resolution lower than 3.0 Å, it was difficult to locate them with confidence (especially the mobile Qh) due to partial occupancy and quinone mobility.

The native XRD data indicate that there are no large-scale rearrangements of the Mn4CaO5 cluster and its protein environment between the different states (dark, 2F, 3F and 3F′) in PS II. This is in line with Mn EXAFS data, which suggests that the largest possible changes in Mn–Mn distances on Mn oxidation state do not change within 250–300 s after the third photo-excitation, in agreement with previous UV–Vis and XAS data. The long delay before the onset of Mn oxidation/reduction in the 3Zox state is consistent with the earlier kinetic results. The Mn oxidation state does not change within 250–300 s after the third photo-excitation, in line with the earlier XAS studies. The observation of an oxygen radical species within this time period cannot be excluded by our data (as no Mn oxidation would be involved). The Mn oxidation state does not change within 250–300 s after the third photo-excitation, in line with the earlier XAS studies. The observation of an oxygen radical species within this time period cannot be excluded by our data (as no Mn oxidation would be involved).
agreement with previous studies. Structural changes that are large enough to access with the current XRD resolution of 4.5–5.2 Å were not observed in the OEC, surrounding amino acid residues, or the quinone sides on the $S_0$ state formation, which implies that the structural changes in the OEC are within the order of $\leq 0.5$ Å. Interestingly, our RT structural data clearly show the presence of several features—although not interpretable at the present resolution—in the m$F_o$–m$F_c$ difference electron density maps, indicating structural differences in the RT XFEL data compared with the previous SR cryogenic structural models. Future improvements in the crystal quality and data, especially the anomalous signal, will also allow us to use the XFEL approach to resolve the sequence of the important structural and electronic changes during the $S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_0$ transition, providing unprecedented experimental insights into the mechanism of photosynthetic water oxidation.

Determination of the total O$_2$ produced per PS II complex. Determination of the total O$_2$ produced per PS II complex and flash number requires the absolute cell content of the MIMS signal collection be calibrated by the injection of known volumes of air-saturated water into the MIMS cell. This value was used to determine the micromoles of O$_2$ produced by PS II by the illumination with 3 flashes using 7 μl per fibre measured in a silica capillary (ID = 75 μm, OD 160 μm), with a flow rate of 0.5 μl min$^{-1}$ and a flash frequency of 4 Hz. To account for different losses of O$_2$ out of the MIMS cell and the light intensity during the light flashes, a loss factor was determined separately by measuring the O$_2$ content of a PS II sample that was illuminated inside a gas tight syringe by 50 consecutive Xenon lamp flashes (2 Hz frequency, in the presence of acceptors) and either directly injected into the MIMS cell or first flowed through the capillary set-up to test for the flash measurements into the collection syringe and then injected into the MIMS cell.

After correcting the O$_2$ amount obtained in the 3F experiment by the loss factor, this number was then divided by the μmole of PS II reaction centre, which resulted in $0.73$ O$_2$/RC (Fig. 2a). As three consecutive flashes are required to produce one molecule of oxygen in a dark-adapted PS II reaction centre, a calculated factor of 3 O$_2$/reaction centre was used for the 3F experiments. The above number of 73% oxygen yield directly translates into light saturation. For 100% light saturation, we would expect a value of 73% (O$_2$), as even under stationary conditions an average ‘moss’ of 10% occurs due to charge equilibria within PS II.

Sample injection and illumination at CXI. Samples were injected into the CXI instrument chamber using an electrospray liquid microjet. Aliquots of 50–150 μl were placed into a 1 ml flow cell attached to the capillary with a flow rate of 0.5 ml min$^{-1}$.

Methods

Sample preparation. PS II was purified from T. elongatus as described elsewhere. Crystals that were obtained as described in ref. 48 and a seed kit (Hampton Research, CA, USA) were used to produce a PS II seed stock solution in buffer C (100 mM PIPES, pH 7.5, 5 mM CaCl$_2$, 6% (w/v) PEG 2000, 0.03% β-dodecyl maltoside) for microcrystallization of PS II. Microcrystals of PS II were obtained by mixing aliquots of the PS II seed stock solution with PS II solution (chlorophyll (Chl) concentration 4 mM, corresponding to a protein concentration of 40–40 mg ml$^{-1}$) in a 1:4 ratio. Box-shaped crystals (5–10 μm in the longest dimension, 5 μm in the shorter dimension) were suspended in buffer C (100 mM MES, pH 6.5, 5 mM CaCl$_2$, 10% (w/v) PEG 2000, 30% (w/v) glycerol). The final concentration of the crystal suspension was determined by measuring Chl concentration of small aliquots of the suspension, dissolved in 80% acetonitrile. The Chl concentration was adjusted between 0.3 and 0.5 mM, corresponding to a protein concentration of 8.5–14 μM (3–5 mg ml$^{-1}$). For solution samples, the purified PS II was resuspended in buffer D (100 mM MES, pH 6.5, 5 mM CaCl$_2$, 0.015% β-dodecyl maltoside, 1.3 M sucrose) to a final protein concentration of 80–90 mg ml$^{-1}$.

Thermolysin was obtained from Hampton Research (CA, USA). Microcrystals of thermolysin were obtained as described previously using PEG2000 as a precipitant.

MIMS measurements. Sample suspensions of PS II from T. elongatus (8 mg ml$^{-1}$) were diluted to 7 mg ml$^{-1}$ Chl with H$_2$O (98%) to give a final enrichment in H$_2$O of 0.12% and final concentrations of 4% salt (150 mM Cl$^{-}$), 85 mM MES and 1.1 M sucrose. No electron acceptors were added. The O$_2$-enriched samples were loaded into a gas-tight Hamilton syringe and pumped through a silica capillary (inner diameter (ID) = 50 μm, outside diameter (OD) 160 μm) into another gas tight Hamilton syringe that collected the sample. Both syringes were filled with the same syringe pump settings. The samples were kept in the dark or very dim green light during all steps, except when illuminated inside the capillary with laser light travelling through one to four optical fibres (400 μm core diameter) directly attached to a region of the capillary with the polyimide coating removed. This set-up directly mirrors the in-capillary illumination set up for the CXI experiment (see Fig. 1a and below).

The oscillation pattern of PS II crystals was obtained in the same way, but the experimental details were as follows: the PS II crystal suspension was concentrated to 3.2 mM Chl and was then diluted with H$_2$O (98%) to 2.5 mM Chl to give a final enrichment of 21.3%. The final concentrations of other additions were 5 mM CaCl$_2$, 1.2 M sucrose and 110% PEG 2000. The capillary that was used to conduct these experiments had an ID of 100 μm and an OD of 360 μm. A Nd:YAG laser (Continuum Inlite II-20, 532 nm, 7 ns pulse width) was used for sample illumination. To obtain a stable output intensity of 7 μl per fibre (intensities of individual flashes may vary by ± 5%), the laser was operated continuously at 20 Hz. The illumination periods were set with the help of a fast shutter (SH05 operated with SC10 Controller; both from Thorlabs), while the flash frequency was controlled via the Q-switch divider (20 or 10 Hz). The oxygen produced was quantified by injecting the illuminated sample into a membrane-inlet cell containing 600 μl water, connected via a Si membrane (Mem 213) and a collimating trap (dry ice/ethanol) to an isolation ratio mass spectrometer. The O$_2$ formed during illumination was detected with excellent S/N ratio as the non-labelled H$_2$O$_2$, the mixed labelled H$_2$O$_2$ and double-labelled O$_2$O species. To obtain a flash pattern, the light-induced yields for O$_2$ production (detected at m/z = 34) obtained with (−1) illuminations were subtracted from that with 3 illuminations (Fig. 2b). For the first flash, the background 32O$_2$ signal of a non-illuminated sample was subtracted. Each measurement was repeated twice (deviation of the points was within 10%).
...the resolution dependence both of the multiplicity and of the detector size). The signal was recorded on a 140 k CSPAD, located below the cryostat surrounded by He as a heat-exchange gas. The ESRF storage ring was run in 16 bunch mode with ring currents between 60 and 90 mA. The incident beam was monochromated and tuned to 6,750 eV using the 111 reflection of a pair of cryogenically cooled Si crystals. The beam size was 1.0 (h) × 0.2 (v) mm² and the beam position on the sample was changed after 1 s of X-ray illumination.

Computational facilities. Over a 5-day period, 114-TB data were collected at LCLS, grouped into five 12-h shifts. Data were processed immediately to assess their completeness and quality. However, as the data size exceeded the processing capacity of the 480-core Linux cluster available at LCLS, arrangements were made to access an additional 1,000 Linux cores at the National Energy Research Scientific Computing Center (NERSC). Transfer of the data from SLAC to NERSC was made using the large CSPAD at LCLS's XRD data processing. XRD data were recorded using the large CSPAD at LCLS's CXI instrument16, and processed using cctbx.xfel25,26. A dark-current image (pedestal) was subtracted from each image before data reduction. An initial trapezoid step was evaluated, retaining only those images containing 16 or more strong, low-resolution Bragg spots as determined by the Spotfinder procedure26,27. However, it was found that this step rejected some useful data; thus, it was ultimately omitted from the data processing protocol. Indexing (determination of the unit cell and crystal orientation) was performed with the LABELIT implementation36 of the Rossmann DPS algorithm59,60, and was guided by supplying the known unit cell61,62. Where more than one crystal was exposed in the same shot, indexing was attempted on the two most dominant lattices8. The number of images or lattices retained after each processing step is detailed in Supplementary Table 1.

Crystal orientations determined by LABELIT were optimized by minimizing the positional difference between the observed Bragg spots and those predicted by the model. Orientational models were further refined so that minimal perturbations were needed to exactly fit the observed Bragg spots to Bragg's law, under the simplifying assumptions of a perfect crystal lattice and a monochromatic beam. Differences between this idealized model and the actual set of observations were needed to exactly fit the observed Bragg spots to Bragg's law. Under the simplifying assumptions of a perfect crystal lattice and a monochromatic beam, the positional difference between the observed Bragg spots and those predicted by the model. Orientational models were further refined so that minimal perturbations were needed to exactly fit the observed Bragg spots to Bragg's law, under the simplifying assumptions of a perfect crystal lattice and a monochromatic beam.

Intensities were integrated by summation within a spot mask derived from nearby strong spots atop a planar background26,83, and corrected for polarization15. Intensity variances, σ²(I), were derived by counting statistics66 and a coarse estimate of the detector gain. Error estimates from each diffraction pattern were accounting for the partiality fraction of each observation. Images with intensity variances correlated poorly (<10%) with those of the reference model were rejected, as were images that deviated from the reference unit cell lengths (10%) or angles (2°), or that did not obey the expected symmetry. Multiple measurements with the same Miller index were merged by averaging and the error was modelled by propagating the σ(I) values in quadrature. The resolution cutoff for the merged data sets was determined from the resolution dependence both of the multiplicity and of the detector size. The correlation coefficient of semi-data sets merged from odd- and even-numbered images9. The expected contribution of the anomalous signal to |F| was estimated using the Web server of the Biocomb Molecular Structure Center at University of Washington (http://skuld.bmcs.washington.edu/scatter/AS_index.html).

Phasing and refinement. As a starting PSIL model, we used PDB IDs 3bz1 and 3bz2 (ref. 12, modified to include all atoms in the OEC based on the high-resolution structure 3arc13, and re-refined against the 3bz1/3bz2 deposited amplitudes in phrenix.refine67. The structure was then refined to a single copy of the PSII complex and the processed data sets were phased by molecular replacement in Phaser18. Refinement of coordinates and B-factors was performed in phenix.refine66 with two-fold non-crystallographic symmetry, with the distances between heavy atoms in the OEC restrained to the values determined by EXAFS20. Simulated annealing omit maps were generated with the OEC atoms set to zero occupancy, with harmonic restraints20 applied to the OEC and surrounding atoms; the default parameters of a starting temperature of 5,000 K and 100 K steps were used. Isomorphous difference maps were generated using phenix.fobs_minus_fobs_map. Structures and maps were aligned using the PHENIX structure comparison tool. All structure figures were created in PyMOL 1.2.

The thermolysin structure was solved by molecular replacement using PDB ID 2thi27 with metals and waters removed, rebuilt using the PHENIX AutoBuild wizard20 and refined in phrenix.refine. Simulated annealing omit maps were generated as for PS II, with Zn and Ca occupancies set to zero.

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Author contributions
U.B., V.K.Y. and J.Y. conceived the experiment; U.B., J.Y., V.K.Y., J.K., R.A.-M., J.M., A.Z., N.K.S., G.J.W., S.B., A.R.F., D.M. and M.J.B. designed the experiment; R.T., J. Hellmich, G.H., R.C., D.D., M.I., C.G., J.K., A.L., B.L.-K., S.G. and A.Z. prepared samples; S.B., J.E.K., M.M., M.M.S. and G.J.W. operated the CXI instrument; M.J.B., H.L., R.G.S., J.K., J.M., B.L.-K., S.G., R.T. and C.G., J.Hellmich and G.J.W. developed, tested and ran sample delivery system; S.K. and J.M. conceived, set up and performed O2 evolution measurements; R.C., R.A.-M., E.G., J.U. and P.G. collected SR XES of PS II; R.A.-M., J.K., R.T., B.L.-K., S.G., T.-C.W., D.S. and J.Y. developed and tested XES set-up; R.A.-M., A.B., U.B., M.J.B., S.B., R.C., R.J.G., P.G., C.G., S.G., G.H., J.Hattne, J.Hellmich, J.K., J.E.K., A.L., H.L., B.L.-K., D.M., M.M., J.M., N.K.S., M.M.S., R.G.S., C.A.S., D.S., R.T., T.-C.W., G.J.W., V.K.Y., J.Y. and A.Z. performed the LCLS experiment; J. Hattne, N.E., R.J.G., A.B., R.A.-M., J.K., C.A.S., P.H.Z., M.M., P.D.A. and N.K.S. developed new software and/or processed and analysed data; D.E.S. arranged computer access; J.K., J.Y., J.M., N.K.S., U.B. and V.K.Y. wrote the manuscript with input from all authors.

Additional information
Accession codes: The X-ray crystallographic coordinates and structure factors for structures reported in this study have been deposited at the Protein Data Bank (PDB), under deposition numbers 4TNL (thermolysin), 4TNH (PS II dark state), 4TNJ (PS II 2F), 4TNI (PS II 3F) and 4TNK (PS II 3F'). These data can be obtained free of charge from www.pdb.org.

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