Supplementary Information

Supplementary Discussion of Methods

Isolation and crystallization of Photosystem II

Photosystem II (PSII) was isolated from *Thermosynechococcus elongatus* as described in [1] with the following modifications. The samples were frozen after the ion exchange chromatography step and batch precipitation/crystallization of PSII was performed four times in decreasing concentrations of PEG 2000, the last purification step was performed at LCLS directly prior to growth of microcrystals.

Standard crystallisation methods, such as vapor diffusion and hanging drop, have become the dominant techniques for the growth of protein crystals in the past decade. These methods have been optimized for very small volumes of protein that are currently not useful for serial femtosecond crystallography (SFX). Batch crystallisation has been successfully used for the growth of large PSII crystals for standard crystallography [1] and crystallisation in batch method can be easily scaled up for large protein volumes. Growth of very small PSII crystals (approximately 1 μm) using the batch method, at high yield, requires high super-saturation conditions to enhance nucleation. This leads to a very broad size distribution of crystals, visible crystal defects, and a coexistence of crystals and amorphous precipitate.

The growth of PSII microcrystals for data collection was performed using a free interface diffusion technique that had been adapted to a batch method for a higher yield. In this approach, nucleation is initiated at the interface between the high density precipitant solution and the lower density protein solution, containing PSII detergent micelles. The protein solution was prepared by dissolving the PSII precipitate from the 4th precipitation

step (see above) in solubilisation buffer A-sol (100 mM Pipes pH 7.0, 5 mM $CaCl_2$, 10 mM tocopherol, 0.03% ß-dodecylmaltoside (DDM), glycon >99.9% purity), adjusting the concentration of chlorophyll (Chl) to 0.5 mM. The crystals were grown in batch experiments in 15 mL Falcon tubes by adding precipitation buffer to a final concentration of 100 mM Pipes pH 7.0, 5 mM $CaCl_2$, 10 mM tocopherol, and 10-17% PEG 2000 to the protein solution. The optimal PEG and protein concentration was experimentally determined for each protein batch separately, in small scale experiments, before the remainder of the protein was crystalized on site at LCLS directly prior to data collection. All precipitation steps and the crystallisation were carried out in darkness to avoid pre-illumination of the crystals. The PEG precipitant solution was added to the PSII solution at a rate of approximately 20 μL per second. The slow addition of the PEG precipitant, with a higher density than the protein solution, led to a two phase system, where the precipitant solution gathered at the bottom of the tube with a small mixed zone in between the top protein layer and the bottom PEG layer. Once the crystals formed and reached a sufficient size they sedimented into the precipitant solution and formed a pellet at the bottom of the tube. As the precipitant solution did not contain protein, the crystal growth stopped (see Extended Data Fig. 1a-e). To further ensure that crystal growth had been terminated, the supernatant was removed after 48 hours and a stabilization buffer (100 mM Pipes pH 7.0, 5 mM $CaCl_2$, 10 mM tocopherol, 20% PEG 2000) was added. This buffer also served as the running buffer for delivery of the crystals to the X-ray interaction region during the TRSFX experiments. The supernatant was saved and later used once crystals reached sufficient size, as it continued to crystallize at a significantly slower rate, due to the decreased protein concentration. The crystallization progress was

monitored closely by taking 1 µL samples at regular intervals to determine crystal size by dynamic light scattering (DLS) (See Extended Data Fig. 1d). Crystals were harvested and directly used for the SFX experiments after they reached a size of around 1 µm. While DLS provides the size distribution of the particles, it cannot discriminate between amorphous and crystalline particles. The crystallinity of the samples was therefore monitored by SONICC, which detects nanocrystals as small as 100 nm[15]. Extended Data Fig. 1a-e show the crystallization method and results of crystal characterization experiments.

All handling steps with the crystals were performed in dim green light to limit exposure. After growth and stabilization, crystals were stored in complete darkness. All steps thereafter were done in the dark.

The plastoquinone derivative PQ_{decyl} was not added to the crystals until the beamtime in June 2012 and therefore was not a part of the double-flash (i.e., putative S_3) experiments. Jesse Bergkamp synthesized PQ_{decyl} in the labs of Ana L. Moore and Thomas A. Moore at ASU. It contains the same head group as plastoquinone but the 15 units of the isoprene tail were replaced by an *n*-decyl chain to improve solubility. We have independently determined that addition of PQ_{decyl} maintains full oxygen evolving activity of PSII under continuous illumination for several minutes. A plastoquinone molecule is located in the quinone binding pocket Q_B in S_1, after two laser excitation flashes, reaching the putative S_3-state, the natural plastoquinone (PQ) becomes double reduced to PQ^{2-}, which takes up two protons and leaves the binding site as plastoquinol PQH_2. The empty binding pocket is then re-populated by $PQ_{decyl,}$ before the third laser flash induces the next charge

separation event to reach the S_4-state (see Fig. 1a of the main text for the S-state scheme that also features the reduction state of the quinone in each of the S-states).

Characterization of microcrystals by SONICC and DLS

All crystal samples were characterized via two methods, second order nonlinear imaging of chiral crystals (SONICC) [2]. The SONICC and DLS experiments were performed using 24 well VDXm plates for data collection from 1 µL suspension of the crystals. The reservoir was filled with 500 µL of precipitation buffer to prevent evaporation of the 1 µL hanging drop containing the crystals. The crystals were monitored by the SONICC system, at 200 mW laser power for an exposure time of 1 second. In the SONICC technique, the crystals were excited by two femtosecond infrared laser pulses of 1064 nm leading to second harmonic generation. The SONICC signal was detected at 532 nm. The dynamic light scattering experiments were performed in 1 µL hanging drops. Our DLS instrument is equipped with an infrared laser at 785 nm to avoid excitation of the pigments in PSII during the measurements. Attempts to conduct DLS measurements on PSII samples with a red laser, as used in most commercial DLS instruments, failed due to the strong absorption of red light by the chlorophylls in PSII, which strongly diminished the signal. For each sample, 10 measurements were performed with 20 seconds of data collection per measurement. The viscosity of the buffer solutions was determined experimentally by calibration with 140 nm polystyrene beads. The crystal size distribution was found to be around 1 µm (see Extended Data Fig 1d).

EPR characterization of the S-state transition

Electron paramagnetic resonance (EPR) has been used extensively to determine the progression of PSII through the S-state cycle [3,4]. Using this technique, quantification of the S_2-state is determined by the multiline signal (MLS), a signature of only this state of PSII. Protein solutions, used for PSII crystallization, were cycled through the S-states by multiple single flash laser excitation (1-3 flashes) at room temperature. The samples were flash frozen directly after laser excitation in liquid nitrogen and the yield of multiline signal was interrogated by EPR at low temperature. Please note that the EPR experiments were performed under conditions that were as similar as possible but not identical to the LCLS experiments (for example the EPR data collection required freezing in glycerol, while SFX data are collected at RT without glycerol addition). As the S-state yield is an estimate, the double-flash state is indicated as "putative S_3-state" in the manuscript. Prior to illumination, glycerol was added to samples as a cryo-protectant, yielding a final concentration of ~30% by volume. This resulted in a final PSII concentration of 1.8 mg Chl/ml (2 mM). Dark adaption was performed prior to the EPR experiments, therefore the PSII samples were initially, predominately in the S_1-state. We did not attempt to get all the PSII into the S_1-state, using pre-illumination flashes in the presence of artificial electron acceptors followed by dark adaptation (as described by [4]) as the natural mobile plastoquinone (1 Q_B per reaction centre) would leave the binding site and consequently be lost in the pre-illumination phase.

For flash illumination at room temperature (20°C), a Continuum Surelite EX Nd:YAG laser was used with a second harmonic generator yielding 532 nm, 8 ns, 1 Hz, and ~380 mJ (fluence of ~10^3 mJ/cm^2) pulses. Low-temperature X-band EPR spectra of the flash-

frozen samples were recorded using a Bruker EMX X-band spectrometer equipped with a X-Band CW microwave bridge. The sample temperature was maintained at 10 K by an Air Products LTR liquid helium cryostat during collection of the EPR spectra. Spectrometer conditions were as follows: microwave frequency, 9.48 GHz; field modulation amplitude, 25 G at 100 kHz; microwave power, 31 mW.

Dark-adapted samples of both PSII solutions and crystal suspensions (frozen without illumination) contained a small percentage (typically 10%) of multiline signal. To determine the maximal possible yield of the MLS, the dark adapted, frozen PSII samples were illuminated at 190 K (dry ice/ethanol bath) for 20 minutes. Please note that the S-state cycle stops at the S_2-state at low temperature (190K) [3], therefore all photoactive PSII reaction centres can be brought into the S_2-state by low temperature continuous illumination. The illumination of the frozen crystal suspensions was performed in 2 minute intervals with the maximum MLS signal intensity achieved within the first 2 minutes of continuous illumination. We observed that the presence of glycerol affects the intensity of the MLS. Solutions and crystals to which no glycerol was added exhibited lower MLS intensity after continuous illuminations.

Extended Data Fig. 1f shows the EPR spectra of PSII samples, which were excited by 1 or 2 laser flashes at room temperature, followed by flash freezing in the dark. In addition the graph also shows the control experiment where dark-adapted frozen PSII was continuously illuminated at 190 K to achieve the maximal S_2 yield. A miss parameter α=9.7% was obtained by fitting the MLS intensities as a function of laser flashes, see Extended Data Fig. 1g. Samples exposed to three flashes were also included. The data

evaluation indicates that with two flash illuminations at least 70% of the PSII reaction centres have reached the S_3-state under these conditions.

The transition rates are comparable to results of EPR studies on spinach PSII by Styring et al., who published transition rates of max 75% under conditions that were highly optimized for maximal yields of S-state transitions [4].

CXI instrument setup and sample delivery for time-resolved femtosecond crystallography data collection on PSII crystals in the double-flash state.

Time resolved femtosecond X-ray crystallography data were collected at the Coherent X-ray Imaging (CXI) instrument [5] at the Linac Coherent Light Source (LCLS) [6] at SLAC National Accelerator Laboratory. The PSII crystals were delivered to the interaction region with the FEL beam as a suspension of crystals using the gas focusing liquid injector described in [7,8,9]. The injection process was improved by the invention of an anti-settling device [10], which also was modified to permit temperature control of the sample. Stainless steel syringes containing the crystal suspension (pre-filtered through 10 µm stainless steel filters from IDEX) were mounted on a rotating holder, which was cooled with a Peltier element to 10°C. This setup maintained the crystals at their growth-temperature until their delivery to the FEL interaction region by the gas focusing jet. The glass capillary nozzle tips were polished to allow for visible laser excitation of the crystals in the nozzle tip. A black coating upstream of the nozzle tip prevented pre-excitation of the crystal suspension upstream of the optical laser interaction region. The gas focused liquid jet had a diameter of 4 µm at the intersection with the X-ray focal area of 2 μm^2 FWHM (full width at half maximum) using the CXI instrument. Data were

collected at the X-ray photon energy of 6.0 keV (2.05 Å) with X-ray pulse duration of approximately 50 femtosecond. The X-ray diffraction patterns were detected on a Cornell-SLAC Pixel Array Detector (CSPAD) [11,12]. The detector consisted of 64 panels, each 194×185 pixels tiled to span approximately 1728×1728 pixels with gaps between the tiles and approximately 19 cm along one side.

Double laser excitation of Photosystem II crystals

PSII was excited by two subsequent optical pump-laser pulses from a diode-pumped, frequency-doubled Nd:YLF laser (Evolution-30, Coherent), emitting visible laser pulses at a wavelength of 527 nm. The laser was fibre-coupled from a table outside the experimental chamber, channeled into the chamber and onto the head of the liquid jet injector [7, 8]. This wavelength provided a good compromise between transmission and absorption of light in the PSII crystals to ensure approximately homogeneous excitation throughout the crystals (the size of crystals was approximately 1 μm), as identified by DLS (See Extended Data Fig. 1d). The optical double pulse was produced by an active Q-switch with "on times" chosen such that the pulse energies of both laser pulses match. This resulted in pulse lengths of 90 nanoseconds and 150 nanoseconds for the first and second pulses, respectively this was done to maintain the total number of photons incident on the crystal same. The laser was focused to an area of approximately 400 μm in diameter with a flat top profile and aimed at the transparent tip of the nozzle, about 100 μm upstream from the x-ray interaction region. The laser beam diameter and aim point were chosen based on the desired pump-probe delays and calculations of sample flow profile and flow speed inside the capillary (average speed of 85 mm/s for 50 μm inner diameter of the nozzle) and in the jet (12 m/s for the 4 μm jet). This allowed for

illumination of the crystals at the tip of the nozzle and in the liquid jet. It also ensured that crystals probed by X-rays were first exposed to both optical laser pulses for the "pumped" measurements (see Fig. 1b, c of the main text). The molar extinction coefficient was determined from dissolved PSII crystals at 527 nm, which was then used to calculate the absorption of PSII crystals with approximately 1 μm path length. From these parameters, it was calculated that a minimum fluence of 2.3 mJ/cm^2 (or a pulse energy of 3 μJ for a 400 μm diameter spot) was required to excite every PSII complex in a crystal of 1-2 μm diameter.

During the experiment, the laser pulse energy was monitored using a power meter placed at a 50:50 beam splitter on the laser table (50% of the energy going into the experiment). The energy of the laser pulse transmitted to the sample was calculated by the previously measured transmission of the entire fibre setup from the 50:50 beam splitter to the final lens in the injector (including fibre couplings and feed-through) and using 20% as a conservative estimate for the light transmission through the optically transparent end of the nozzle to the sample. That transmission could not be measured directly, so it was indirectly estimated. The laser pulse energy was chosen to correspond to approximately 6 μJ per pulse at the sample, i.e., three times what is required to optimally pump a 1-2 μm PSII crystal at 527 nm [4]. The desired timing of the optical laser pulses with respect to the X-ray pulses from LCLS [6] were achieved by using the LCLS/CXI event generator and event reader (EVG/EVR) system that provided precise timing signals less than 1 μs before every X-ray pulse (or every other X-ray pulse) and a SRS DG645 delay generator to produce properly timed double trigger signals for the laser Q switch. The time delay between the two optical laser pulses was set to be 210 μs corresponding to three times the time constant for the S_1 to S_2 transition of 70 μs [13]. The time delay between the second

optical pulse and the X-ray pulse was 570 μs to allow the Oxygen-Evolving Complex (OEC) to complete the S_2 to S_3 transition (3 times the time constant of 190 μs for the S_2 to S_3 transition) [13]. As the electron transfer between Q_A to Q_B takes place in the 200 to 400μs time range [14], (depending also on the organism and oxidation state of Q_B) the delay time of 780 μs between flash 1 and the X-ray pulse represents a reasonable minimal delay time aimed at following both the processes at the donor site in the OEC and the acceptor site at the Q_B binding site. The uniform change in unit-cell dimension in the double-flash experiment (putative S_3-state) and its reversion in the triple flash experiment (putative transient S_4-state) provides an independent indication for significant and uniform progression of the OEC through the S-states.

In order to monitor the delay between the pump beam and the probe beam, we separately measured the response of two photodiodes to the optical and x-ray excitation with an Acqiris digitizer. One photodiode was exposed to stray optical light on the laser table and the other to x-rays scattered from the sample jet at very high angle. This was necessary because of the disparity in the response of the diodes to each signal near the interaction region - i.e., at the required optical and x-ray fluences needed to perform the experiment, the signals could not be discriminated when using a single photodiode. The delay between the signals introduced by the measurement of the optical light on the laser table was measured at the beginning of the experiment by equalizing the signals from each beam near the x-ray interaction point. That constant was used to calculate a calibrated delay time with sufficient precision for our experiment using the digitizer trace.

The LCLS timing signal triggered the preceding optical laser pulses for every other X-ray pulse allowing us to acquire alternating diffraction images from “dark” (ground state) and “double-flash” PSII crystals in the putative S_3-state, i.e., SFX data were collected with a

frequency of the X-ray pulses of 120 Hz while the frequency of the pump laser pulses was 60 Hz (Fig. 1b, c). This approach minimizes other sources of error that might occur from systematic error. With this setup, alternating "dark" and double excited "illuminated" images were collected at 3600 dark images and 3600 illuminated images per minute. In addition to this alternating experimental scheme, dark run data was collected while the 527 nm pump laser was switched off (see Extended Data Table 2b for data statistics of dark only and alternate dark/light runs). Representative diffraction patterns of the S_1 and the double-flash data sets are shown in Extended Data Figs. 2a, b.

Processing and evaluation of data with the Cheetah and CrystFEL programs

A total of 5,528,071 raw diffraction frames were collected from Photosystem II microcrystals in January 2012. The raw diffraction data (XTC format) were pre-processed by the *Cheetah* software package, (http://www.desy.de/~barty/cheetah, Barty et al, in preparation) and then analyzed in the software suite CrystFEL [15]. Examples of diffraction patterns of the PSII crystals are shown in Extended Data Fig. 2a for the dark (S_1) state and in Extended Data Fig. 2b for the double-flash (putative S_3) state.

The first step of pre-processing involved dark current subtraction from each diffraction frame and masking of dead, hot, cold, and saturated pixels. It also included masking of the low-resolution scattering from the liquid jet and the detector panel edges. Local background correction step was applied to the raw diffraction frames during their pre-processing in Cheetah (http://www.desy.de/~barty/cheetah) (publication Barty *et al*. in preparation). Each diffraction frame was analyzed and identified as a crystal "hit" only if it contained 25 or more peaks with the intensities of at least 400 analogue-to-digital units.

The locally background-corrected patterns collected in the alternating mode were sorted into two data sets for the dark and double-pumped hits based on signals from a photodiode and video camera in the experimental chamber. Comparison between the dark data sets from the alternating light/dark runs and complete dark runs showed no difference in unit cell constants, or indexing rates, therefore the dark data sets were merged. The final data sets contained 71,628 "hits" from the dark state data set and 63,363 "hits" from double-flash data sets. The triple-flash data sets, leading to the putative transient S_4-state of PSII (collected during a separate beamtime in June 2012), were collected in alternate runs with the frequency of 120 Hz of the LCLS X-ray pulses and 60 Hz laser excitation frequency. The same laser excitation scheme as described before was used to reach the S_4-state. The third laser excitation, reaching the S_4-state, was achieved by triggering a third laser excitation 527 nm laser pulse 570 μs after the second laser excitation pulse. This third pulse was triggered by a second 527 nm laser installed in the CXI hutch, which excited the jet through a separate hole in the shroud. The delay time between the third laser flash and the FEL X-ray pulse was 250 μs. The data sets from the January 2012 beamtime are designated as dark (A) and double-flash (A) and the data sets from the June 2012 beamtime are designated as dark (B) and triple-flash (B) (see Extended Data Table 2a). The dark (B) and triple-flash (B) data sets contained 33,373 and 32,190 hits, respectively. The "hits" for all data sets were passed as separate sets to the CrystFEL software suite [15] for auto indexing with MOSFLM, using the orthorhombic unit cell dimensions of PSII from *Thermosynechococcus elongatus* (PDB code 1FE1) [16] within a tolerance limit of 6%, 5%, 5% for the reciprocal axes of *a, b, c* respectively for the S_1-state. Similarly, the tolerance limit of 8%, 5%, 5% were used for the reciprocal

axes of *a, b, c* respectively for the double-flash and triple-flash states. After indexing, the 4 data sets were handled separately and the "indexing rates" (fraction of "hits" which could be successfully indexed) were 48% for dark (A), 29% for double-flash (A), 35% for dark (B), and 39% for triple-flash (B). Extended Data Table 2a, b shows the indexing statistics as well as the unit cell constants for all 4 data sets. The unit cell is orthorhombic and shows very significant changes in the unit cell dimensions between the dark S_1 and double-flash data sets (A) state (see Extended Data Table 2 and Extended Data Fig. 3). The most pronounced change is in the dimension of the *a* axis which increases by 3.3 Å. This change in unit cell constants is accompanied by the slight decrease in diffraction quality (5 vs. 5.5 Å resolution) (see Extended Data Tables 1, 2,) and significant lowering of indexing rates. This change in the unit cell dimension is fully reversed to the unit cell constants observed in the dark S_1-state when PSII crystals are excited by three laser flashes, eventually reaching the putative transient S_4-state. Furthermore, the indexing rate for the dark (B) and triple-flash (B) data sets are comparable, with 35% and 39%d, respectively

Multiplicity in the measurements is very important due to the partial nature of all reflections, the variation of the flux in each FEL pulse and the fact that each diffraction pattern is collected from a different crystal. The triple-flash data set, with 12,500 diffraction patterns, is sufficient to accurately determine the unit cell constants. However, this is borderline for the determination of accurate structure factors and therefore, further data evaluation has been limited to the dark (S_1) and double-flash data sets.

The dark (S_1) and double-flash (putative S_3) data sets that were used for structure factor determination consist of 34,554 and 18,772 indexed patterns, respectively. Our data sets

were merged separately in three dimensions and the structure factors were extracted separately from dark and double-flash data sets of PSII protein using the Monte Carlo method [17], which integrates the snapshots partial reflections from randomly oriented crystals of varying size and shape (see Extended Data videos for a graphical representation of the structure factor amplitudes of the both data sets). Average multiplicities are 684 and 373 in all resolution shells from 19.20 Å to 4.03 Å of the dark (S_1) and double-flash states, respectively (see Extended Data Tables 1a, b).

A comparison of the data statistics of our work with that of Kern *et al.* 2013 [18] is shown in Extended Data Table 2c. Our data sets show significantly higher multiplicity of the data and better correlation coefficients ($CC_{1/2}$) when compared to Kern *et al*., which are indicative of the quality of the merged reflections.

The internal consistency of the SFX data is expressed as R_{split} [15] . For the calculation of R_{split} the sets of images of a data set are split into two random halves, and the structure factors are calculated separately for each half. The difference between the amplitudes of each of the structure factors of (hkl) plane between the two half data sets is used to estimate the convergence of the full dataset as given by R_{split}. i.e., an R_{split} of 0.22 means an estimated 22% error of the structure factors of the full data set to the "true" dataset. (The two half datasets would have agreed to 0.22 * sqrt(2).)

R_{split} was calculated for the dark and double-pumped data sets separately. As expected, with an increasing number of indexed patterns, R_{split} decreases as the data set reaches higher multiplicity and completeness (see Extended Data Fig. 4). The average R_{split} values over all resolution shells were 0.07 for the dark and 0.09 for the double-pumped data sets.

Structure determination

The data for the dark (S_1) and double-flash (i.e., putative S_3) state of PSII were handled as two completely separate data sets for the whole data analysis process. After processing the raw serial femtosecond X-ray crystallographic data, the final structure factors from dark and double-pumped data sets were passed to the CCP4 Software package [20] .

Molecular replacement

Molecular Replacement [19] was carried out by the PhaserMR program, which is a part of the CCP4 suite [20] using the PSII X-ray structure at 1.9 Å resolution of Umena and co-workers (PDBID: 3ARC) [21] as the search model, which was modified by removing waters, detergents, lipids, and alternative conformers of the amino acids. We used monomer 1 of the PSII dimer (where the monomer 1 subunits are labelled with capital letters and small letters are used for monomer 2 in 3ARC model) as search model for molecular replacement to solve the phase problem by using the program phaser (version 2.5.3). After we found monomer 1, we repeated the search for monomer 2.

Refinement

Both structures were refined by *phenix.refine* [22] using rigid body refinement, where each C-alpha chain of each protein subunit was considered as a rigid body. The cofactors were also considered as rigid bodies. During the rigid body refinement, we considered only translational refinement and not rotational refinement of the rigid entities. So, the RMS bond angle was not refined. We used the original B factors from the 3ARC model because B-factor refinement is not useful at the given resolution of 5.0 Å. After three refinement cycles, R-factors for the dark state at 5.0 Å of R_{work} of 0.260 (R_{free} = 0.262) were observed. The R-factors for the double-flash state at 5.5 Å, are R_{work} of 0.280 (R_{free} = 0.290). Refinement statistics are shown in Table 1 of the main text.

All figures displaying structures were made using PyMOL (DeLano Scientific; http://www.pymol.org).

Calculation of electron density maps

The electron density maps for dark and double-flash data sets were generated using the FFT program from the CCP4 suite. The omit maps, defined by Bhat, were calculated using "Omit" (CCP4 suite) [23, 24] where the OEC was removed from the MR solution. These ($2F_o - F_c$) omit maps were calculated using experimental data and the MR model excluding the OEC before applying refinement in order to avoid model-bias. For superimposition of these two omit maps from dark and double-flash states respectively, the following steps were carried out. First, using the omit maps as inputs, new sets of coordinate files (pdb files) were generated from the MR solutions of dark and double-flash states separately, so that each of the two omit maps fits the model, using the Molrep program in CCP4 suite. Second, the modified coordinate files for the dark and double-flash states, outputs of Molrep program, were opened, and the superimposed coordinates were saved in the coot program[25]. The double-flash state coordinate file was considered as the moving object and the dark state coordinate file as the fixed object. As a result, coot provided Euler angles and translational coordinates (x, y, z values) for this superposition. Third, using these Euler angles and translational coordinates as rotational operator with opposite sign, the double-flash state omit map was rotated using the MAPMASK program in the CCP4 suite. Because the unit cell constants of the dark and double-flash electron density maps differ, they are in different frames of inertia, which we have to take into account for the overlaying process. The rotated double-flash state

omit map (output of MAPMASK program) was moved over the superimposed coordinate file. The same procedure was applied to the dark state omit map aligning with the superimposed double-flash coordinate file using MAPMASK program in the CCP4 suite. Examples of the electron density maps are shown in Extended Data Figs. 5-7.

Calculation of Simulated Annealed omit maps

The solutions from the molecular replacement for the PSII dimer for the dark and double-flash states were used for the calculation of the Simulated Annealed (SA) omit maps [26]. For each of the PSII coordinate files of the MR solutions, the OEC was removed and then the resulting PSII coordinate file was used for calculating the SA-omit map with a starting temperature of 5000 K using the "AutoBuild create omit map" program from the Phenix suite (version 1301 dev) [27]. The SA omit maps of the OEC in the dark state (S_1) and the double-flash (putative S_3) state are shown in Figs. 3a, b of the main text and also see Extended Data Fig. 8 for the dark state SA omit map from a different view-point.

Supplementary Discussion of Results

Unit Cell Increase:

The conformational change and associated unit cell changes may be caused by dissociation of the mobile plastoquinone PQ_B from the Q_B binding pocket after double-flash excitation, when PSII may reach the S_3-state (see Fig. 1a). The structural changes leading to the difference in unit cell constants are likely most significant at the stromal side of PSII where the quinone bindings sites are located. To avoid structural

heterogeneity at the acceptor side by partial re-occupation of the Q_B binding site, no quinone was added to the crystals for the double pump experiments. We thereby may have "trapped" PSII in the double flash experiment in the putative S_3-state conformation with an empty Q_B binding pocket. In order to transition from $S_3 \rightarrow S_4$, an electron acceptor must replenish the empty Q_B binding site. Therefore, the plastoquinone derivative $PQ_{decyl,}$ which diffuses into the Q_B pocket, was added to the crystals used for the triple-flash excitation data set. With the Q_B binding site re-occupied, the change in unit cell constants is reversed.

Structural changes of the OEC:

In light of new results on theoretical modelling of the OEC [28,29, 30, 31, 32, 33] we further examined the SA-omit maps in the dark and double-flash states for differences in the metal cluster that can be detected even at low resolution and discuss the results here in light of recent computational and spectroscopic studies on the metal cluster. The changes in the density of the Mn_4CaO_5 metal cluster are suggestive of an increase of the distance between the cubane and the 'dangler' Mn and a distortion of the cubane in the S_3-state. The observed electron densities are compared in Fig. 3a and b of the main text with the recent theoretical studies of Isobe and co-workers [30], shown in Fig. 3d (see main text), who predicted a "breakage" of the dangler Mn from the cubane cluster in the S_3-state. Additionally, EXAFS data constrains the extent of the movement of the dangler Mn relative to the cubane [34,35]. The increase in distance could allow for the binding of the second substrate water molecule between the dangler Mn and the Mn_3CaO_x cubane. The presence of a substrate water molecule between the dangler Mn and the distorted cubane in the higher S-states, has also been predicted to be essential for the catalytic mechanism

in a recent DFT model of the full catalytic S-state cycle, including modelling of the substrate water exchange [29, 36]. In addition to the elongation, the overall dimensions of the Mn_4CaO_5 cluster appear to condense in the double-flash data set that may represent the putative S_3-state. This may include shrinking of the distance between the Ca^{2+} and the 3 Mn in the distorted cubane. EXAFS studies on PSII, where the Ca was substituted with Sr, showed significant changes in Mn-Mn or Mn-Sr distances in the S_3-state [37], which were interpreted to indicate the distance between Mn and Ca would shrink in the S_3-state. Our experimental findings suggest a shrinking of the Mn_4CaO_5 cluster in double-flash state, which supports the hypothesis of a condensation of the Mn_3O_xCa cubane part of the Mn_4CaO_5 cluster in S_3 [30]. Models of Mn-oxygen cubane compounds show an increased distance between the Mn and O atoms in the cubane at lower oxidation states (+2 and +3) due to the Jahn-Teller (JT) effect [33,38]. Distances derived from a recently published model Mn-O and Mn_3O_xCa cubane structures [39] indicate that Mn-O distances depend on the oxidation states of the Mn-ions: the average Mn^{+2}-O distance is 2.2 Å, the average Mn^{+3}-O distance is 2.0 Å and the average Mn^{4+}-O distance is 1.8 Å. Two models have been proposed on the basis of X-ray absorption and emission spectroscopy, one describes the S_3-state as Mn (+3 +4 +4 +4) and the other proposes Mn (+4 +4 +4 +4) [40,41]. In the model where all Mn ions have reached the Mn^{+4} oxidation state, a significant shrinking of the dimension of the cluster is expected due to the lack of the JT distortion with the average Mn-O distance being reduced to 1.8 Å [38]. The shrinking of the overall dimensions of the metal cluster, supported by our maps of the double-flash state, appears to be in agreement with the studies on model compounds. This indicates that the JT

distortion diminishes in the putative S_3-state during progression of the S-states cycle when all Mn reach their +4 oxidation states [39].

The SA-omit maps of the dark (S_1) and the double-flash (putative S_3) states may be also indicative of changes in the protein environment of the Mn_4CaO_5 cluster. While the electron density map in the dark S_1-state overall follows the protein backbone of the 1.9 Å structure, larger perturbations of the protein environment of the cluster are visible in the double-flash state. The double-flash state electron density map may suggest a movement of the loop which connects the transmembrane helices C and D at the lumenal site (the CD loop, including D170) away from the metal cluster and a movement of the AB loop (connecting the transmembrane helices A and B) into closer vicinity to the cluster, which may allow D61 to become part of the ligand sphere of the metal cluster. While this interpretation of changes in the protein environment of the cluster is highly speculative at the given resolution, it could explain the results of mutagenesis studies on PSII. Although the mutation of D170A (which coordinates the dangler Mn and the Ca in the 1.9 Å structure of PSII) has no strong effects on the oxygen evolution function [42,43], less than 15% of the oxygen evolution function remains in the D61A mutation [44,45]. This mutagenesis result was difficult to rationalize because D61 is found only in the second ligand sphere of the OEC in the 1.9 Å structure [21]. However, our SA-Omit electron density map of the metal cluster in the double-flash state shows a connection to the protein electron density in close vicinity to D61 (see Fig. 3b). This finding may provide a first indication that D61 may serve as a ligand to the dangler manganese in the higher S-states. While details of the conformational changes cannot be unravelled at the current resolution of 5 Å, the comparison of the dark and double-flash state SA omit maps

provide an indication that the protein ligand sphere of the Mn_4CaO_5 cluster may undergo significant changes when the OEC reaches the double-flash (putative S_3) state.

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