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Crystallization of Photosystem II for Time-Resolved Structural Studies Using an X-ray Free Electron Laser

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Abstract

Photosystem II (PSII) is a membrane protein supercomplex that executes the initial reaction of photosynthesis in higher plants, algae, and cyanobacteria. It captures the light from the sun to catalyze a transmembrane charge separation. In a series of four charge separation events, utilizing the energy from four photons, PSII oxidizes two water molecules to obtain dioxygen, four protons, and four electrons. The light reactions of photosystems I and II (PSI and PSII) result in the formation of an electrochemical transmembrane proton gradient that is used for the production of ATP. Electrons that are subsequently transferred from PSI via the soluble protein ferredoxin to ferredoxin-NADP⁺ reductase that reduces NADP⁺ to NADPH. The products of photosynthesis and the elemental oxygen evolved sustain all higher life on Earth. All oxygen in the atmosphere is produced by the oxygen-evolving complex in PSII, a process that changed our planet from an anoxygenic to an oxygenic atmosphere 2.5 billion years ago. In this chapter, we provide recent insight into the mechanisms of this process and methods used in probing this question.

1. INTRODUCTION

Two and a half billion years ago oxygenic photosynthesis evolved resulting in the drastic oxygenation of Earth's atmosphere which led to an explosion of biodiversity and, eventually, the evolution of higher organisms. Photosystem II (PSII) is crucial to oxygenic photosynthesis. As indicated by the close homology of the oxygen-evolving complex (OEC) across many species, it has only substantially evolved once and the core functions and structure have been maintained through billions of years of evolution. PSII is large membrane protein complex made up of 19 protein subunits and over 50 noncovalent cofactors. During photoactivation, the OEC of this massive complex proceeds through a five state photochemical reaction, the Kok cycle, over the course of which four charge separations occur (Fig. 1). One electron and one proton are extracted in each of the charge separation events, leading to two water molecules being deconstructed until the formation of dioxygen each cycle. PSII is able to oxidize water, driven by visible light and catalyzed by earth abundant metals at a low overpotential of +1.1 V (Wydrzynski & Satoh, 2006). However, with such a high redox potential, it operates at the limit of the stability of biomolecules. The unraveling of the mechanism of water splitting in PSII is one of the major goals in bioenergetics as it would open the door to the development of synthetic oxygen

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evolving systems that combine the major catalytic features of PSII with the stability of artificial systems.

The first static structure of PSII was determined to 3.8 Å in 2001 (Zouni). The resolution was increased subsequently (Ferreira, Iverson, Maghlaoui, Barber, & Iwata, 2004; Loll, Kern, Saenger, Zouni, & Biesiadka, 2005; Umena, Kawakami, Shen, & Kamiya, 2011) and the highest resolution structures are now available at the near atomic resolution of 1.9 Å (Suga et al., 2014). In order to understand the catalytic mechanism at work, time-resolved studies with microsecond time resolution are needed to explore the multiple photoexcited states in the Kok cycle (Renger, 2012). Traditional macrocrystallography is unable to unravel the structure of the OEC in its different oxidation states due to site-specific radiation damage of the OEC by X-ray photoreduction. X-ray absorption fine structure spectroscopic studies have indicated that the manganese ions in the OEC possess a high propensity to site-specific X-ray-induced reduction (Allakhverdiev et al., 2005). A bias may also arise with cryo-cooled crystals as data are collected far removed from biologically relevant temperatures. The recently developed method of serial femtosecond X-ray crystallography (SFX) has been used to investigate the structure of selected photoexcited states of PSII (Kern et al., 2013, 2014; Kupitz, Basu, et al., 2014). Very recently, X-ray free electron lasers (XFELs) have also been used to determine the first high resolution undamaged dark structure of PSII based on serial data collection on large cryo-cooled crystals (Suga et al., 2014).

Fundamentally, SFX is based upon using highly coherent, extremely brilliant, femtosecond hard X-ray pulses to collect thousands of X-ray diffraction snapshots on a hydrated stream of small crystals at room temperature (Chapman et al., 2011), enabling diffraction information to be obtained before the ensuing Coulomb explosion destroys the molecules including each measured crystal (Neutze et al., 2000). In this way, radiation damage is “outrun” in the data collection and samples can be probed at more biologically mimetic conditions compared to traditional protein macrocrystallography. Furthermore, using a fresh crystal to produce each diffraction pattern, time-resolved photoexcitation processes can be probed by coupling an optical pump probe to the sample prior to interaction with the X-ray beam (Aquila et al., 2012; Neutze & Moffat, 2012; Spence, Weierstall, & Chapman, 2012). For the first time, irreversible processes are now able to be probed in a time-resolved experiment due to the use of discrete crystals for each diffraction pattern. A delay time between the optical pumping and beam interaction as well as the number of times the sample is flashed with light can be modulated to explore different transitory states. Recent work has advanced the knowledge of the structure and further advancements will ultimately be able to resolve the mechanism of photosynthetic water splitting and oxygen evolution. The method of time-resolved SFX (TR-SFX) has the potential to lead to a “molecular movie” of photosynthetic water splitting in the future. In this chapter, recent advancements of methods developed for TR-SFX of PSII will be summarized and compared.

2. ISOLATION OF PHOTOSYSTEM II

All structures solved so far from PSII are based on PSII isolated from the thermophilic cyanobacteria *Thermosynechococcus elongatus* (*T. elongatus*) (Ferreira et al., 2004; Kern et

al., 2013; Kupitz, Basu, et al., 2014; Loll et al., 2005; Zouni et al., 2001) and *Thermosynechococcus vulcanus* (*T. vulcanus*) (Kamiya & Shen, 2003; Suga et al., 2014). As PSII undergoes the process of photodamage and repair (Aro, Virgin, & Andersson, 1993), the reproducible growth of large quantities of the cyanobacteria is an essential prerequisite of all functional studies. Our group uses a large 122L photo-bioreactor that has been developed together with the Pulse Institute that controls temperature, pH, CO₂, and air flux and measures cell density which can be coupled to the light intensity for growth of *T. elongatus* for reproducible growth of the cells at low light conditions to minimize photodamage. The complete isolation procedure from cell harvest to growth of crystals is performed in one setting within 48 h under dim green light which involves three recrystallization steps. The methods have recently been published in Kupitz, Grotjohann, et al. (2014).

3. CRYSTALLIZATION FOR STUDIES WITH FELs

Due to X-ray damage, macromolecular crystallography requires very large single crystals of PSII on the order of millimeters in size to allow for a shift of the crystals after each image during data collection. However, even with an extremely careful shift strategy such as applied in Umena et al. (2011), photoreduction is difficult to minimize, leading to a photoreduced structure of the OEC.

SFX studies depend on a continuously fresh stream of small crystals to obtain diffraction before destruction. This leads to the need for size homogeneous nano- or microcrystals (on the order of 500 nm to 5 μm) created using novel crystallization techniques. New methods have been developed by our team using free interface crystallization to allow for reproducible growth of large quantities of microcrystals of PSII with a very narrow size distribution of centered around 1 μm^3 as described in Kupitz, Grotjohann, et al. (2014). We will discuss the different methods used for the growth of PSII microcrystals below in more detail.

3.1 Batch methods and establishing the phase diagram

The batch method describes crystallization through a homogenized solution containing the protein and precipitant. Initial trials to establish knowledge of phase space are easily accessible using this method, owing to the highly controllable and quantifiable solution environment. Seed crystals can be added at different points in phase space and monitored to map out the phase diagram. Seed crystals will dissolve in the nonsaturated zone, grow without additional nucleation in the metastable zone, and induce nucleation of additional crystals in the nucleation zone. Particularly important is that the amount of induced nucleation scales with distance from the border between the metastable and nucleation zones, permitting optimization of conditions with respect to yield, crystal size, and homogeneity.

3.2 Free interface diffusion

Figure 1 shows a schematic of the free interface diffusion (FID) method developed for the growth of PSII nanocrystals that have been used for our TR-SFX studies (Kupitz, Grotjohann, et al., 2014). The method is based on the idea that in order to develop a so-

called “shower” of small crystals, one must rapidly access a point in the nucleation zone of phase space so that a high nucleation rate is achieved. This provides an alternative to vapor diffusion experiments where it is often difficult to control crystal growth at high supersaturation.

The following procedure describes a way to access the phase space. This construction also results in the deposition of crystals into a pellet, which can be easily collected and allows for further quenching and control of crystal density. FID has been used for growth of larger crystals in a traditional setup, where crystallization occurs inside a capillary (Ng, Gavira, & García-Ruiz, 2003). By the use of thin capillaries, diffusion is slowed so larger crystals are grown along a concentration gradient (McPherson, 1991). In contrast, for growth of nanocrystals rapid nucleation is desired. The precipitant, which has a higher density than the protein solution, is slowly added dropwise to the protein solution (see Fig. 2). The drops of precipitant passing through the protein solution cause the protein to interact with a high concentration of precipitant. This continues as the dense precipitant forms a layer underneath the solubilized protein. The interface between the two solutions allows for rapid nucleation. Once the crystals reach a certain size, they sediment into the precipitant where their growth is quenched. Centrifugation immediately after the formation of the interface not only results in crystals that can be seen within 30 min but also results in a more homogenous size distribution of crystals due to a rapid density separation. Thereby, the FID method provides an intrinsic quenching of crystal growth and a uniform size distribution for PSII crystals of $1\ \mu\text{m} \pm 500\ \text{nm}$ (Kupitz, Grotjohann, et al., 2014).

3.3 Quenching

Once a yield of crystals has been determined to be at an acceptable size and distribution, it is desirable to quench any further growth prior to diffraction or during sample delivery. While the FID setup has a temporary quenching mechanism, it is not sustainable due to the slow onset of equilibrium and complete mixing of the layers. It is important to remove the free protein that could lead to growth of existing crystals, further leading to clogging of injectors and a broader size distribution of crystals. Once the crystals form a pellet, the supernatant is removed and replaced by stabilization buffer that contains 1.25 times of all solutes in the crystallizing precipitant solution. This ensures the stabilization of existing crystals, both in the absence of free protein and through the presence of a more thermodynamically unfavorable solute environment, enabling a strong preference for PSII molecules to remain in the solid crystal phase.

3.4 Quantification of natural plastoquinone and addition of PQ_{decyl}

In order to investigate conformational changes at both the acceptor and donor site of PSII and to allow multiple laser excitation steps, it is important to verify the quinone content of the PSII in the crystals. PQ_B is a mobile electron carrier and light exposure must therefore be avoided during all preparation steps to ensure high occupancy of the QB-binding site with PQ. We have determined the PQ content of our crystals using high-pressure liquid chromatography (HPLC) with a (C-18) column after each PEG 2000 precipitation step. The protein was then subjected to a pigment extraction using acetone according to the protocol from Patzlaff and Barry (1996). The presumed ratio of chlorophyll *a* to PQ is 76:4 at full

quinone occupancy in *T. elongatus*. The area under each peak was integrated to obtain a ratio between chlorophyll *a* and PQ in the extracted sample. From this ratio, a percentage of PQ_B occupancy was calculated from an average value taken from three HPLC runs. This resulted in occupancies of 91.8% before initial crystallization, 88.4 after the first recrystallization, 86.4 after the second recrystallization, and 81.1% after the third recrystallization step.

After two optical events, Q_B becomes doubly reduced to PQ²⁻ and leaves the binding site as PQH₂, and thus needs to be replaced for further oxidation to occur in order to reach the S₄ state. However, PQ is extremely difficult to obtain by synthesis or purification due to poor solubility caused by the long isoprene tail. As a substitute, a derivative of PQ with the same head group but an *N*-decyl chain instead of the isoprene tail, referred to as PQ_{decyl}, was synthesized. This PQ_{decyl} was added to the crystals so that it could repopulate the binding site after the departure of the native PQH₂. Thus, the addition of the PQ_{decyl} allows for the S₄ state to be reached even when the protein is not in its' natural membrane environment.

4. DETECTION AND CHARACTERIZATION OF NANOAND MICROCRYSTALS

4.1 Optical microscopy

During all crystallization trials, drops taken from an individual experiment can be imaged using optical light microscopy, by which crystals >1 μm can be detected. However, one must be cautious drawing conclusions from optical microscopy (OM) alone since the size of the crystals are on or past the edge of what is visible and reliance on OM alone cannot identify nanocrystals <1 μm. One of the most useful methods in OM is the use of polarized light to check for birefringence since protein crystals often possess refractive anisotropy. This can help to distinguish between crystalline protein and amorphous precipitate, provided crystals are >1 μm. Another caution should be mentioned that many salts are also birefringent and, since the birefringence signal will scale with the size of the crystal, crystals that are on the order of 1 μm or less will not be recognizable as such by birefringence.

4.2 Ultraviolet fluorescence microscopy

The use of ultraviolet fluorescence microscopy (UVM) allows the confirmation of protein in the crystals via tryptophan fluorescence. This is complimentary to OM when trying to determine whether or not a birefringent signal comes from a salt or protein crystal. Diffuse signal can also indicate undersaturated conditions or the presence of free protein amongst crystals. In the general case of non-SONICC active protein crystals (see Section 4.3) that may have low birefringence or have a birefringent salt as a precipitant, discrete spots in a UVM image can provide an alternative indication of crystals.

4.3 Second-order nonlinear imaging of chiral crystals

The second-order nonlinear imaging of chiral crystals (SONICC) technique is an ideal method for detecting crystals of a chiral molecule with non-centrosymmetric crystals, which is common among protein crystals (Wampler, Begue, & Simpson, 2008). When a substantially intense electric field is produced by a laser pulse, molecular dipoles are induced. In the case of a chiral crystal, these induced dipoles are anisotropic on their

potential energy surface and allow the sampling of nonlinear, even numbered higher order polarizability terms such as the second-generation harmonic (frequency doubling) (Haupt & Simpson, 2011). Enhanced signal can be measured at half the wavelength of the incident pulse, indicative of chiral crystals due to constructive interference provided by the ordered lattice. PSII is crystallized in space group of $P2_12_12_1$ which is SONICC active and provides positive confirmation of crystals too small to image optically, distinguishing them from amorphous precipitate or identifying them in a visibly clear drop. Protein crystals as small as 100 nm in size can be detected with SONICC. Second harmonic-generated signal was measured at 532 nm, indicative of a two photon process that is only enhanced by the presence of crystals with anisotropic unit cells and is negligible otherwise.

4.4 Dynamic light scattering

Dynamic light scattering (DLS) allows the calculation of particle size and size distribution using a temporal autocorrelation function of the scattered light signal over time in tandem with the Stokes–Einstein equation for particle radius. This comes from the stochastic Brownian motion of particles resulting in a time-dependent scattering intensity caused by interference with the surrounding particles. The first-order autocorrelation function is given by Eq. (1) as a function of the scattering radius q with delay time τ being parameterized. The diffusion coefficient, D_t , can then be identified and further used to calculate the hydrodynamic radius, r , assuming a sphere with known viscosity, η , at a temperature T (Eq. 2). Through computational Fourier decomposition, multiple signals can be identified in the raw data leading to measurement of size dispersion. Thus, once other methods have been employed to confirm the existence of crystals, DLS provides the ability to monitor size and homogeneity at various time intervals during crystallization:

$$g(q;\tau) = e^{-q^2 D_t \tau} \quad (1)$$

$$D = \frac{k_B T}{6\pi\eta r} \quad (2)$$

5. TIME-RESOLVED CRYSTALLOGRAPHY OF PSII USING FELs

The TR-SFX approach using FELs allows for the determination of the structure of undamaged biomolecules at room temperature, as diffraction occurs before destruction takes place (Barty et al., 2012). Furthermore, time-resolved studies can be performed where a reaction is initiated by light or rapid mixing, even on irreversible processes due to the serial delivery of the single crystals in a liquid jet where each femtosecond X-ray pulse hits a new crystal.

Time-resolved studies in crystallography were pioneered with the Laue method which uses a relatively polychromatic “pink” beam and large crystals to study light-induced reversible reactions. Pioneering work has been done with the p21–GTP complex, myoglobin, and PYP (Rajagopal et al., 2005; Schlichting et al., 1990; Šrajer et al., 2001). However, Laue crystallography cannot be used for the study of the S-state cycle of PSII due to the X-ray-

induced reduction of the metal cluster of the OEC. Further limitations are the limited light penetration of the large crystals, prohibiting a uniform population of transitory states. SFX overcomes these problems and opens a new window of opportunity for time-resolved studies toward molecular movies of biomolecules at work.

5.1 Considerations for PSII in TR-SFX

For time-resolved studies, it is important that a sufficient population of the protein molecules must be in the same state when the crystal is probed by the X-rays in order to elucidate changes in the electron density of conformationally active localities. Thus, for light-induced time-resolved experiments, it is imperative that crystals be small enough so that a high majority of the molecules in the crystal are excited by a saturating laser flash. Furthermore, when processing serial data, it is important that Bragg diffraction intensities be comparable between. This leads to the need for size homogeneous nano- or microcrystals (on the order of 500 nm to 5 μ m) to allow maximal uniform excitation from the optical pump laser.

Recently, the dark state of PSII was investigated at the FEL at SACLA by a group led by Jian-Ren Shen. In this study, they collected FEL data on PSII crystals using an alternate fixed target approach whereby they solved the first high resolution undamaged dark structure of PSII based on data collection of very large single crystals at cryogenic temperatures. The dark structure was determined at 1.9 Å resolution and showed smaller distances between Mn atoms of the metal cluster of the OEC compared to the first high resolution structure of PSII based on data collection at synchrotrons (Umena et al., 2011). In this incredibly vast experiment, data were collected on 336 individual large crystals of millimeter size with femtosecond X-ray pulses at the FEL in SACLA. To minimize progression of X-ray damage, data were collected under cryogenic conditions with a defocused beam (1 μ m). With the FEL's relatively large beam focus, the crystals had to be translated 50 μ m between each shot.

While this breakthrough work led to the first undamaged high resolution structure of the dark state of PSII, time-resolved studies, however, will be very difficult using this setup as uniform light excitation cannot be achieved with large single crystals. Additionally, data collection at cryogenic temperatures would not allow progression of the S-states beyond the S₂ state which is reached after a single laser excitation.

When the PSII is excited, light is captured by a large antenna system and the excitation energy is transferred into the center of the complex, where charge separation takes catalyzed by the primary donor P680. The charge is passed through an electron transfer chain from P680 through chlorophyll a, a pheophytin, the plastoquinone PQ_A and finally to the terminal acceptor plastoquinone PQ_B. After two charge separation events, PQ_B is doubly reduced to PQ²⁻, picks up two protons, and leaves the binding site as plastoquinol PQH₂.

Once PQH₂ departs, it is subsequently replaced by another PQ_B from the PQ pool located in the photosynthetic membrane. P680⁺ is concurrently reduced by extracting one electron at a time from two substrate water molecules bound at the OEC via the redox active tyrosine. After four electrons have been extracted in subsequent charge separation events, oxygen is

evolved. The OEC consists of a cubanoid $\text{Mn}_4\text{O}_5\text{Ca}$ cluster which undergoes four corresponding oxidation events and cycles through the Kok cycle. In the absence of light, PSII's ground state is S_1 state where one positive charge has already been accumulated in PQ_B . The oxidation states of the four manganese atoms in the OEC is currently under debate but one likely scenario consists of oxidation states of (+3+3+3+4) for the S_0 state, (+3+3+4+4) for the S_1 ground state, (+3+4+4+4) for S_2 , (+4+4+4+4) for S_3 , and (+4+4+4+5) for S_4 . In order to ensure that the desired excited states (S_2 and S_3) are being reached, it is necessary to verify the enzymatic activity (oxygen evolution) and quinone exchange.

5.2 The pump-probe experiment

In the time-resolved SFX experiments, an optical pump laser is used to excite the crystals preceding interaction with the probe XFEL beam. This technique necessitates a laser excitation scheme with the goal to achieve a maximum excited population amongst PSII molecules in the crystals in each pump pulse and allowing for uniform evolution of transition states between each pump. Two different setups have been developed for TR-SFX studies on PSII.

In the experimental scheme described in Kupitz, Basu, et al. (2014), the crystals are delivered to the FEL beam in a fast running jet (10 m/s) at ambient temperature and hydrated in their mother liquor. The experimental setup is depicted in Fig. 3. The optical laser pulses were triggered by the linac coherent light source (LCLS), meaning that the time delay between the flashes is known exactly and is independent of the flow rate of the liquid jet. The fast running jet uses high amounts of sample at a flow rate of 10 $\mu\text{L}/\text{min}$. However, it ensures that the sample is fully replenished before the next FEL pulse arrives, eliminating the possibility of upstream excitation or damage. Data are collected simultaneously in pulse by pulse alternating light and dark sets, where the pump lasers are triggered with a frequency of 60 Hz synched to the 120 Hz LCLS FEL pulses. To reiterate, this manifests as one snapshot being a “dark” snapshot with the next one being “light” snapshot and so on, resulting in 60 dark images and 60 light images collected per second. This ensures that all variables during data collection are identical for the light and dark datasets. Data were also collected without any laser excitation and comparison shows that the data of the purely dark runs and the alternating dark/light runs are identical. The laser excitation scheme is shown in the bottom of Fig. 3. Delay times of 210 μs between flashes 1 and 2 and 560 μs between flash 2 and the “probing” with the FEL pulse were used, corresponding to three times the measured time constants of the OEC progression from the S_1 state to the S_2 state and from the S_2 state to the S_3 state (Dekker & Van Grondelle, 2000). Data in the literature for the electron transfer between PQ_A and PQ_B greatly vary and are in the range of 200–800 μs . Future planned studies will extend the time points to up to 2 ms.

Ideal time delays between flashes should be in the range between 200 μs and 2 ms to study the conformational changes associated with electron transfer at the acceptor site and oxidation of the Mn_4CaO_x cluster at the donor site. A variation of time delays, including longer time delays up to 2 ms and an improvement in resolution of the structural model, are important to the resolution of conformational changes at atomic detail in any future work.

An alternative approach used by Kern et al. (2013, 2014) for TR-SFX studies. In this approach, the crystals are delivered to the FEL beam in a slow-running jet where the sample is exposed to light by flowing across multiple windows in the nozzle. In this setup, the time delays between the first sets of laser excitations are dictated by the flow rate of the sample in the nozzle and only the last flash is triggered by the incoming FEL pulse. While this sample delivery method has the advantage of low sample consumption, it is limited in that the time delay for the first set of flashes is determined by the flow rate, which is often not constant and also varies within the nozzle as there is an order of magnitude difference in the flow rates of the center and sides of the nozzle. This limits the setup to long time delays between the flashes with an average time delay between the first flashes on the order of 500 ms. This number is only a rough estimate as the flow rates have not been directly determined and were instead estimated from volume filled into the reservoirs and the time the sample ran out (see Kern et al., 2014). Regardless, these long time delays inhibit realizing the conformational changes at the acceptor site as PQ_A^- is oxidized by side reaction with oxygen in 2–3 ms before arrival of the second electron (de Wijn & van Gorkom, 2001).

In summary, the most commonly successful sample delivery method for TR-SFX to date is the gas dynamic virtual nozzle which uses amplified liquid pressure and gas focusing to deliver the sample. It has not only been used for the TR-SFX studies on PSII but also formed the basis for the first TR-SFX study that reached atomic resolution, using the photoactive yellow protein as a model system (Tenboer et al., 2014). Using the lipidic cubic phase (LCP) as a delivery media allows for minimal sample consumption with flow rates on the order of nL/min (Weierstall et al., 2014). This media has also been shown to successfully support membrane protein crystals with datasets solved with <0.5 mg. However, the crystals must be grown in the LCP, necessitating possibly new conditions, and the optical density of the material and slow flow rate is prohibitive toward pump-probe studies. Another recent approach to SFX sample delivery is through the use of a nanoflow electrospinning microjet as described in Sierra et al. (2012). This method conserves sample by use of an applied electrical current to induce an electrospinning jet, as opposed to gas focusing, and the use of a viscous media such as glycerol to control droplet formation which allows a flow rate for PSII crystals around 3 μ L/min. While this balances the need for sample conservation with a jet that is able to be optically pumped, the nature of the jet only allows for delay times on the order of seconds and concern arises with regards to structural artifacts generated by the applied voltage on the protein. A primary drawback is sample consumption with typical flow rates of 10–15 μ L/min but this allows access to the microsecond time range for pump-probe experiments and avoids any possible upstream scattering excitation.

5.3 Evaluating PSII SFX data

For Kupitz, Basu, et al. (2014), the program Cheetah (Barty et al., 2014), developed specifically for SFX data, was used for background correction and hit-finding. The patterns were then indexed and merged using the *CrystFEL* software suite (White et al., 2012) and refined with the *Phenix* software suite (Adams et al., 2002). In SFX each diffraction snapshot represents a thin slice through reciprocal space, resulting in each measured reflection representing only a partial measure of scattering factor. Furthermore, the intensity between individual X-ray shots varies by more than 200%. In light of this, the determination

of accurate structure factors requires a high multiplicity of Bragg intensities with a recommended minimum of 50, a sharp contrast to traditional synchrotron crystallography. A multiplicity of >600 for the dark dataset and >300 for the double flash datasets (putative S_3 state) have been achieved in the Kern et al. experiment (2014). Kern et al. (2014) applied a program described for data evaluation. A key difference between this program and Cheetah is that it uses resolution to select images, leading to low multiplicity of the data in the higher resolution shells. By contrast, the hits are selected in Cheetah according to a threshold of spots at a selected signal-to-noise ratio.

5.4 Structural changes of PSII in the Kok cycle

In Kupitz et al., the structure of PSII was solved at 5.0 and 5.5 Å for dark and double-excited datasets, respectively. Large changes were detected in the unit cell constants between the dark S_1 state and the double flash putative S_3 state, which are reversed in triple flash experiments.

Despite the large changes of the unit cell constants, the overall dimensions of PSII do not increase as shown in the overlay of the transmembrane helices in Fig. 4D. However, larger differences are detected in the acceptor side loop regions and the nonheme iron coordinated thereby (Fig. 5). After PQH_2 is formed and leaves the Q_B -binding site, an empty binding site ensues which could trigger the changes of the loops structures and the expansion of the unit cell constants. The fact that all crystals undergo this change in unit cell constant is an independent indication for the homogenous progression of PSII in our crystals through the Kok cycle. This change is reversible as it is reversed in a three-flash experiment where PQ_{decyl} is incorporated to allow for the binding site to be filled before the third flash arrives.

In order to detect conformational changes of the OEC and avoid phase bias simulated annealed omit maps were calculated of the OEC and its protein environment (see Fig. 6A and B). These omit maps tentatively show changes in the OEC and surrounding environment. From the omit map shown in Fig. 6B, an elongation of the Mn_4CaO_x portion of the OEC can be seen in the S_3 state with respect to the S_1 dark state. This may allow for the second substrate water molecule to bind between the “dangler” Mn_4 and the Mn_3CaO_4 cubane-like structure during the S-state transition. This is in agreement with hybrid density functional theoretical modeling (Isobe et al., 2012), which has shown that the substrate water has a probable minimum on its potential energy surface when coordinated by the Mn_4 and modulated by the O_5 (Fig. 6C). Furthermore, a decrease in $Mn-Ca^{2+}$ distances seen through extended X-ray absorption fine structure (EXAFS) spectroscopy, pointing to a change in the character of the Mn_4-O_5 bond that would occur during elongation.

The observed electron density changes shown in Fig. 6 agree with the recent theoretical studies of Isobe et al. (2012), who predicted a “breakage” of the dangler Mn from the cubane cluster in the S_3 state. In addition to the elongation of the electron density in the direction of the dangler Mn, the overall dimensions of the Mn_4CaO_5 cluster appears to shrink in the S_3 state, and the distance between the Ca and the 3 Mn in the cluster decreases, as part of the Ca sticks out of the electron density map in the S_3 state. EXAFS studies on PSII where the Ca was substituted with Sr showed very similar spectra in S_1 and S_2 , indicating that no significant changes occur in the Mn–Mn or Mn–Ca distances in this S_2 -

state transition, while significant changes in EXAFS spectra were observed in the S_3 state (Pushkar, Yano, Sauer, Boussac, & Yachandra, 2008), which included the prediction that the distances between Mn and Ca would shrink in the S_3 state. Experimental findings in Kupitz et al. (2014) support a shrinking of the Mn_4CaO_5 cluster in S_3 which would support the hypothesis of a condensation of the Mn_4CaO_5 cluster in S_3 based on the Jahn–Teller (JT) effect which has also been studied in several model Mn compounds (for more details on studies on Mn model compounds, see Yamaguchi et al., 2013 and references therein). Mn–O distances derived from recently published model Mn–O and Mn_3Ca –O cubane structures (Kanady, Tsui, Day, & Agapie, 2011; Mukherjee et al., 2012) indicate that Mn–O distances depend on the oxidation states of the Mn ions. The average Mn(II)–O distance is 2.2 Å, the average Mn(III)–O distance is 2.0 Å and shrinks to 1.8 Å for the Mn(IV)–O distance. Based on X-ray absorption and emission spectroscopy, two models exist for the oxidation states of the Mn_4CaO_5 in S_3 , which is either described as Mn(III)(IV)₃ or Mn(IV)₄ (Dau, Zaharieva, & Haumann, 2012; Yano & Yachandra, 2007). In the model of S_3 where all Mn ions have reached the Mn(IV) oxidation state, a significant shrinking of the dimension of the cluster is expected due to the JT distortion with the average Mn–O distance being reduced to 1.8 Å (Yamaguchi et al., 2013). The shrinking of the overall dimensions of the metal cluster, which is supported by our maps of the putative S_3 state, appears to be the first experimental indication of the role that the JT distortion plays in the mechanism of water splitting (Kanady et al., 2011; Mukherjee et al., 2012).

Changes are also visible in the protein environment of the metal cluster. These are much more difficult to interpret and validate at low resolution than the changes of the metal cluster and have to be confirmed at higher resolution. While the dark state SA-omit map (Figs. 4A and 5A) matches the structural model of dark state (Umena et al., 2011), there are significant changes visible on the SA-omit map of the double flash putative S_3 state. The SA-omit map of the putative S_3 state is suggestive of conformational changes which may indicate movement of the CD loop (including the ligand D170) away from the cluster. If this could be confirmed at higher resolution, it would indicate that Asp170, which provides ligands to both Ca and the dangle Mn in the dark state, may not be a ligand in the higher S-states. The loop between the transmembrane helices A and B (AB loop) may change its conformation so that it moves closer to the metal cluster. A density feature connects this loop at the position of Asp61 to the dangle Mn of the metal cluster in the putative S_3 state. Mutagenesis studies and recent spectroscopic evidence also support the current interpretation. Mutagenesis experiments have questioned Asp170 as a ligand in the higher S-states, as mutants still show 80% oxygen evolving activity and the FTIR spectra are not significantly altered in mutants of Asp170 (Debus, Strickler, Walker, & Hillier, 2005). While Asp61 only serves as a second sphere ligand in the 1.9 Å crystal structure (Umena et al., 2011) mutagenesis studies indicated an important role in the water oxidation process as the S_2 to S_3 transition is blocked in Asp61 mutants (Debus, 2014; Dilbeck, Bao, Neveu, & Burnap, 2013; Pokhrel & Brudvig, 2014; Service, Hillier, & Debus, 2010).

At low resolution an interpretation of changes in the protein environment is challenging and the question may arise: how robust are these changes in the SA-omit map?

Three validation tests have been performed, comprised of the annealing temperature of the SA-omit map (Fig. 7A), splitting of the data for both the dark and light datasets in half and calculation of the SA-omit maps with the split datasets (Fig. 7B), and calculation of the SA-omit maps for the light and dark states using exactly the same structure factors (Fig. 7C). The results in Fig. 7 and the comparison with the SA-omit map in Fig. 6 show that the changes of the metal cluster and its protein environment between the dark and putative S_3 state are visible in all cases.

6. SUMMARY

The structures of PSII in the S_1 and S_3 states were determined at 5.0 and 5.5 Å, respectively, by TR-SFX. The experiments required new developments in crystal growth and crystal characterization so that a high density of microcrystals of PSII of very homogenous size could be achieved. Uniform growth of nano- and microcrystals requires knowledge of the phase diagram and analytical techniques such as SONICC and DLS are crucial to detection and characterization. Using the pump-probe TR-SFX setup, radiation free snapshots of the S-state cycle can be recorded at biological temperatures, allowing for deeper understanding of the enzymatic mechanism behind PSII. Future developments may eventually lead to an evolution from snapshots of transitory states to molecular movies, enabling thorough understanding of enzymatic mechanisms such as PSII. Elucidating the mechanism of photosynthetic water oxidation and oxygen evolution is critical to the understanding the underlying photosynthetic mechanism and holds high potential for applications such as renewable energy and membrane protein bioengineering.

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Figure 1.

Schematic of the Kok cycle showing photoinduced progression from the dark S_1 state to the S_4 state, where oxygen evolution occurs followed by a final photoexcitation returning the PSII to the dark S_1 state. PQ_B is reduced at each excitation, leaving, and being replaced by a fresh PQ_B upon arrival at the S_3 and S_1 states. *Figure originally published in Kupitz, Basu, et al. (2014).*

Figure 2.

Stepwise procedure depicting FID and FID centrifugation nanocrystallization techniques. (A) Shows a minimal mixing layering scheme between the protein (green; dark gray in the print version) and the precipitant (white). This results in few small crystals originating at the interface. (B) Shows the precipitant being dropped through the protein, creating a slightly larger mixing zone than (A), resulting in a large shower of microcrystals. (C) Shows the time progression when the experimental setup depicted in (B) is subjected to centrifugation, expediting the formation of crystals, and causing a pellet of size homogeneous crystals to form. *Figure originally published in Kupitz, Grotjohann, et al. (2014).*

Figure 3.

Diagram of the experimental setup for the time-resolved SFX experiment in which the S_1 and S_3 states are probed alternately at 60 Hz each. The optical pump laser scheme is shown at the bottom, indicating delay times of 210 μs between the first and second pump, and 570 μs between the second pump and interaction with the FEL beam, allowing population of the S_2 and S_3 states, respectively. *Figure originally published in Kupitz, Basu, et al. (2014).*

Figure 4. Electron density omit maps at 1.5σ of the PSII homodimer. (A) The dark (S₁ ground) state, (B) the doubly excited (putative S₃ state), (C) ribbon and loop model of PSII with labeled subunits, and (D) overlay of S₁ and putative S₃ states, revealing conformational changes evolved in progression through the Kok cycle. *Figure originally published in Kupitz, Basu, et al. (2014).*

Figure 5.

Overlay of omit maps for the dark S_1 (green; dark gray in the print version) and double flash (putative S_3 (white) state). (A) View of the transmembrane region of photosystem II along the membrane plane (B) more detailed view of the acceptor site which contains the binding sites for PQA and PQB. Note the nonheme iron (red (gray in the print version) sphere) and the loop regions exhibiting significant conformational changes between the two states.

Figure originally published in Kupitz, Basu, et al. (2014).

Figure 6.

Simulated annealing omit maps of the OEC. (A) and (B) Exhibit the S_1 and S_3 states, respectively, at 1.5σ . In (B), the blue represents the S_1 state, whereas the yellow represents the S_3 state for comparison. (C) Shows the crystal structure from the $1.9 \text{ \AA}$ structure of the OEC from Umena et al. (2011). (D) Shows the proposed S_3 state derived from DFT calculations performed in Isobe et al. (2012). *Figure originally published in* Kupitz, Basu, et al. (2014).

Figure 7.

(A and B) Comparison between SA-omit maps of the two halves of the randomly split dark dataset (S_1) at the contour level of 1.5σ (A) and comparison between SA-omit maps of the two halves of the double-excited dataset (S_3) at a contour level of 1.5σ (B). (C and D) Comparison between two SA-omit maps made at two different start temperatures (2000 and 5000 K) for S_1 state at 1.5σ level (C) and the comparison between the two SA-omit maps made at two different start temperatures (2000 and 5000 K) for S_3 state at 1.5σ contour level (D). (E and F) SA-omit maps calculated at 500 K with the same hkl values at 1.5 contour level for the dark dataset (E) and the double flash dataset (F).