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Enzyme Catalysis

Protein Crystallography Using Free-Electron Lasers: Water Oxidation in Photosynthesis**

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In 2000 Neutze et al. postulated that very intense femtosecond X-ray pulses from a free-electron laser (XFEL) could be used to generate a diffraction pattern for a protein, prior to its obliteration, and coined this approach "diffraction before destruction".[1] This basic principle has recently been demonstrated experimentally and has opened up new opportunities for the study of macromolecular structures and their dynamics.[2] Currently two XFELs are in operation: the LCLS in Stanford, USA, and SACLA at Hyogo in Japan. These XFELs generate extremely short pulses, ranging from a few femtoseconds to 100 fs with a brightness of 10^{12} photons per pulse focused on a sub-micrometer spot; that is, the X-ray brilliance is 10^{10}-fold greater than that from third-generation synchrotrons. The minimum photon wavelength is in the range of 0.05–0.15 nm, and the X-ray pulse rates are 60 (SACLA) and 120 (LCLS) pulses per second.

The advantages of XFEL crystallography include: 1) limited or no radiation damage to the protein during data collection, as the measurement is completed in the fs range; 2) only very small crystals are necessary to obtain a diffraction pattern; 3) data can be collected at room temperature under physiological conditions, which is advantageous for understanding reaction mechanisms and protein dynamics; and 4) time-resolved crystallographic experiments are becoming possible on timescales shorter than current Laue diffraction experiments.[2]

Since the XFEL pulse is extremely intense, the crystals have to be replaced after each shot (Figure 1). For measuring multiple nanocrystals with high throughput, several techniques have been developed.[3] One is jet injection of nanocrystals as a liquid or aerosol, for example, as in LCP (lipid cubic phase) crystallography. LCP crystallization is often applied to membrane proteins. The complete diffraction data set is then obtained by "serial" injection of millions of crystals. Due to the random orientation of the crystals and the varying intensity of each X-ray pulse, special detectors and massive postdata processing are required. Another approach is to use a fixed target such as a large crystal or embedded microcrystals in a film on a translation stage. It is important to note that new structures can be solved by serial femtosecond X-ray crystallography (SFX), which was recently demonstrated using phase determination with single-wavelength anomalous dispersion.[4]

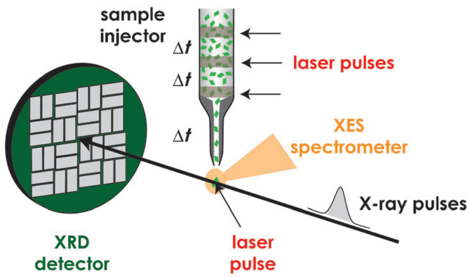

Figure 1. The PS II crystals supplied by a sample injector are illuminated by different laser pulses to create the various catalytic states. X-ray diffraction (XRD) and XES spectra can be measured simultaneously.

In 2011, the first protein crystal structure using the XFEL at LCLS was reported at 8.7 Å resolution. The authors chose photosystem I (PS I), the largest photosynthetic membrane protein characterized to date by standard X-ray diffraction techniques.[5] At present, more than 24 structures have been solved by XFEL (see Table 1 in Ref. [6]), with the highest-resolution structure obtained at 1.8 Å resolution that of thermolysin.[7] The combination of in vivo crystallization (crystals grown in living cells) and XFEL has also been used for the determination of cathepsin B and cry3 A toxin at 2.1–2.9 Å resolution. Herein, we highlight the recent spectroscopic and crystallographic studies of photosystem II (PS II).[7–10]

The special properties of SFX make it particularly useful for the study of intermediates of enzymatic reactions to understand details of catalytic mechanisms. One of the most important reactions in biology, light-induced water splitting, is performed in nature by PS II, a large photosynthetic membrane protein found in all plants, algae, and cyanobacteria. The water-oxidizing complex (WOC) has a high turnover frequency (500 s^{-1}) and turnover number, limited by the lifetime of PS II protein itself (ca. 30 min under normal light) but which is recovered very quickly by an efficient repair

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mechanism. In 2001, highly active PS II was for the first time crystallized and a structure with a resolution of 3.8 Å was obtained.[14] This was a major breakthrough but unfortunately the resolution was too low to provide much information about the WOC.

It took 10 more years before a high-resolution (1.9 Å) structure was obtained by the groups of Shen and Kamiya[13] showing that the WOC consists of a protein-bound Mn_4O_5Ca cluster (Figure 2). It is known that the cofactor's reaction cycle comprises five redox states, S_0 to S_4, where the subscript indicates the number of stored oxidizing equivalents required to split two water molecules into protons and O_2. Light-induced charge separation in PS II is coupled to the Mn cluster by a redox-active tyrosine residue. Two water molecules enter the active site at different times and are subsequently deprotonated (Figure 2) until in the last transient step (S_4) the O–O bond is formed and O_2 is released.[11,12] The oxidation states of the manganese ions in the cycle, and the water-binding and proton-release steps (see Figure 2) have been proposed based on spectroscopic data.[11]

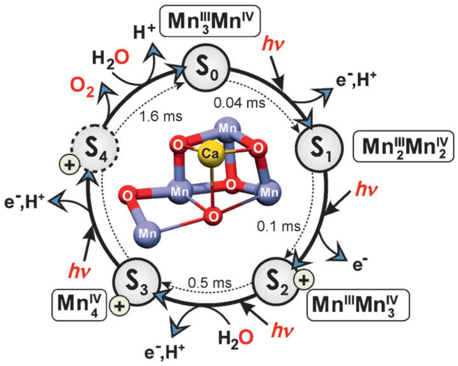

Figure 2. The catalytic S-state cycle of the WOC of PS II[11] driven by four light flashes, showing the electron- and proton-release steps of the reaction, the states in which the substrate water is introduced, the transition times between different intermediate states, and the transient oxidation states of the Mn ions.[11,12] In the center the structure of the Mn cluster from Ref. [13] is shown.

Precise structural data on the intermediate S states of the water-splitting cycle, under physiological conditions, have yet to be obtained. The reasons for this are: 1) the PS II crystals are not very stable at room temperature in the X-ray beam; 2) the preparation and crystallographic characterization of specific intermediate states of the cluster is very challenging; and 3) the Mn_4O_5Ca cluster suffers severe radiation damage under synchrotron radiation,[15] so that the investigation of higher S states is very difficult. Several of these drawbacks can be overcome using SFX techniques at LCLS in Stanford.[9] Of particular interest is the combination of XRD with laser flash excitation of the sample that allows specific S states of the catalytic cycle to be prepared and investigated at ambient temperature. The combination of XRD with X-ray emission (XES) at the beamline opens the possibility of assessing the integrity of the manganese cluster in situ during the SFX experiment. Comparison of the S_1 and S_2 states shows that no major structural change of the cluster occurs, but this conclusion is currently limited by the low resolution of the experiment (5.7 Å).[9]

Recently two independent studies[7,10] of PS II using SFX expanded this work towards the detection of the S_3 state—thus providing a snapshot of the catalyst immediately prior to O–O bond formation. Kupitz et al. observed structural differences between the S_1 and the putative S_3 state resulting from the movement of the outer Mn and two protein loops.[10] They speculated that these changes may result from a substrate water entering the cluster in the S_2 to S_3 transition, a thesis potentially in agreement with recent spectroscopic work of Cox et al.[12] However, in these SFX experiments the resolution is still low (5.0 and 5.5 Å) and the S-state yields were not quantified.

In the second recent study, Kern and co-workers applied SFX to study both the S_3 and S_0 state of the WOC. Their setup, which includes two visible-wavelength lasers for sample excitation, allows the simultaneous detection of XRD and XES. Within their limited resolution (4.5–5.2 Å) these authors were not able to detect structural changes in the WOC during the catalytic cycle, which is in conflict with the work of Kupitz et al.[10] They did, however, detect a transient state within the S_3 to S_0 transition observed using XES.

Both studies demonstrate in a beautiful way the advantages of SFX, namely that diffraction data can now be obtained for very small crystals, without radiation damage, and that transient states of enzymes—even short-lived ones—can be accessed in real time at room temperature. Although drawbacks still exist, the rapid development of these techniques suggests a very promising future for serial femtosecond X-ray crystallography.

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