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How ultrafast X-ray pulses can reveal hidden secrets of photosynthesis

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X-ray-based techniques are extremely versatile and can provide information regarding the atomic arrangement of atoms in a molecule and are often the method of choice for exploring the structures of proteins and their ligands. The photosynthetic splitting of water and evolution of molecular oxygen by plants and cyanobacteria is one of the key reactions in nature, which is catalysed by a metal site in a membrane-bound photosynthetic protein complex. In this article, we will describe how X-ray pulse lasers can simultaneously probe the overall atomic structure of the photosynthetic system and the electronic structure of a catalytic metal site under physiological conditions in real time.

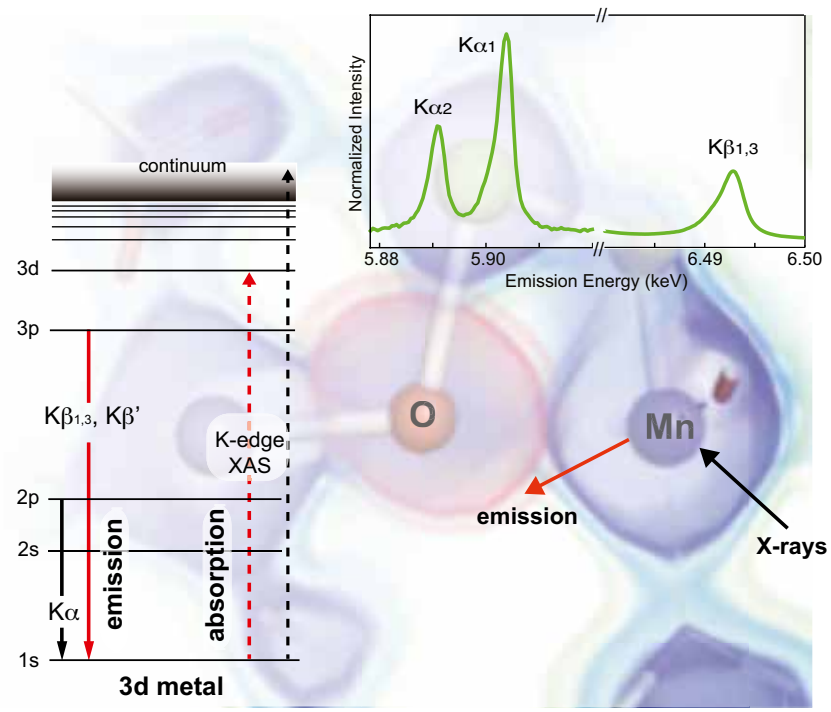

Figure 1. X-ray absorption and emission spectroscopy.
X-ray absorption (XAS) and emission spectroscopy (XES) are element-sensitive techniques for probing the electronic structure of metals. The incident energy can be tuned to the appropriate values for the metal of interest, which in our example is manganese (Mn). XAS and XES are sensitive to the oxidation state and spin state (number of unpaired electrons) on the metal. **Left**: the energy level diagram shows the electronic levels and the transitions involved in X-ray absorption K-edge spectroscopy (1s to 3d and higher levels). X-ray emission Kα and Kβ spectroscopy involves emission from 2p to 1s and 3p 1s levels subsequent to the absorption of a photon from the 1s to the continuum. A typical Kα and Kβ spectrum from Mn is shown on the upper right.

Metal cofactors are integral to the function of many enzymes. In such metalloenzymes, the metal, frequently from the 3d block of transition metals, serves as the site where various chemical reactions such as electron transfer and catalysis take place. This is because of the unique capacity of metals for storing and shuttling electrons by changing oxidation states, controlling spin states, and moving electrons between metals and ligand atoms, i.e., O, N, C, and others that are part of the organic residues that make the protein. A wide variety of approaches have been used to understand how the organic and inorganic groups work together in metalloenzymes in a spatially and temporally controlled manner.

Among these, X-ray-based techniques have been methods of choice to look at molecular structures of protein complexes and their assemblies. The wavelength of X-rays is close to those of the atomic distances in molecules. Therefore, the patterns of diffracted X-rays from crystals can give us information about the atomic arrangement in the proteins. *X-ray crystallography* has been widely applied using laboratory X-ray sources, at synchrotron radiation facilities, and recently at more powerful X-ray sources, such as the X-ray free electron laser (XFEL) facilities.

Alongside this, *X-ray spectroscopy* is a useful method to look at chemical states of metals, such as oxidation states and spin states (Figure 1). It has the capacity to identify specific elements, since X-ray absorption and emission energy depend on the configuration of molecular orbitals of the absorbing element of interest. Only certain transitions are permitted and these transitions are sensitive to changes in the number of electrons in the valence states of metals, the spin state of metals and their interaction with ligands. The spectrum that arises from X-ray absorption or

emission processes can therefore be used as a fingerprint of the chemical state of the metal site.

Can we combine these two powerful methods, X-ray crystallography and X-ray spectroscopy, for the study of metalloenzymes to simultaneously obtain the overall protein structure and the local electronic structure at the active centre (Figures 2 and 3)? Furthermore, we would ideally like to obtain this information at physiological temperatures and in real time to follow the enzymatic reaction, whether it is electron transfer or a catalytic reaction. Fortunately, the X-ray wavelength used for typical crystallography measurements is in the order of 1 Å (angstrom), corresponding to an energy that is above the 1s binding energy of 3d transition metals.

The simultaneous use of X-ray absorption spectroscopy (XAS) with crystallography is challenging as the incident X-ray energy has to be scanned through the absorption edge (a sharp discontinuity in the absorption coefficient of X-rays when the energy is equal to the binding energy of an electron shell in the absorbing element). However, in non-resonant X-ray emission spectroscopy (XES), the excitation energy is not critical as long as it is higher than the absorption edge. Another important advantage of combining diffraction with XES is the fact that the X-ray beam does not have to be highly monochromatic for either technique.

In this article, we describe how optical pump pulses to start the reaction and the ultrafast, ultra-bright XFEL pulses can be used to simultaneously probe the overall atomic structure and the active site electronic structure of photosynthetic systems under physiological conditions, and in real time. These experiments are examples of a changing paradigm of how to study the structure and function of metalloenzymes.

Frozen state or under physiological conditions?

One of the most challenging limitations of X-ray techniques for studying biological systems is the fact that X-rays are highly damaging to those very systems. The interaction of the X-rays with biological matter can change both the native chemical states and the structures of proteins. At the same time, the X-ray interaction with biological matter is, of course, critical; it is the exact reason why X-rays, in particular X-ray spectroscopy, give us important fingerprints to understand the nature of the chemical environment. What we need is to understand the effect of X-ray damage, and thereby control and minimize the degree of change. With biological materials such as proteins, radiation damage mainly occurs due to the migration and diffusion of radicals and solvated electrons to sensitive areas, when X-rays interact with water in the protein. The diffusion

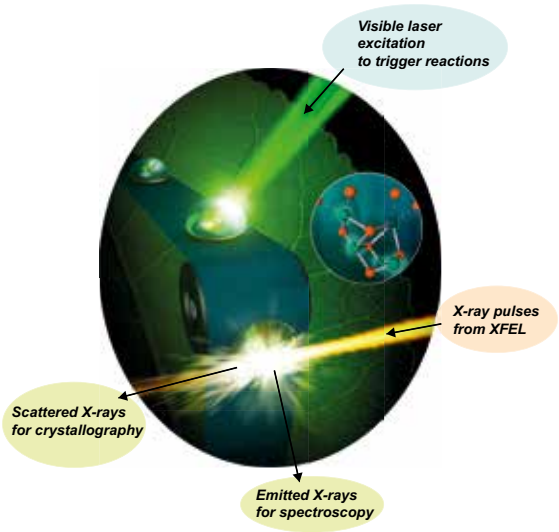

Figure 2. Simultaneous XFEL crystallography and spectroscopy.
The image shows the sample, containing crystals of a protein (in our case PSII), deposited on a moving tape by acoustic methods. The sample is then pre-illuminated by a visible laser to advance the system to a given intermediate state and is then probed by the X-ray pulse from an XFEL. The scattered X-rays downstream are used for crystallography and the photons scattered 90° to the incident beam are used for spectroscopy.

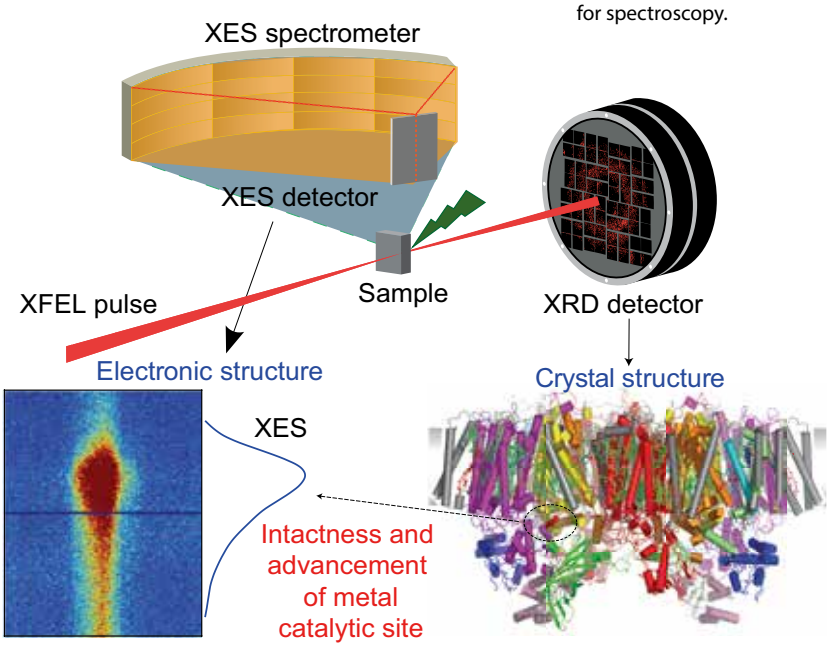

Figure 3. Schematic of XFEL crystallography and spectroscopy experimental set-up.
The schematic (adapted with permission from Fransson, T. et al., X-ray emission spectroscopy as an *in situ* diagnostic tool for X-ray crystallography of metalloproteins using an X-ray free-electron laser, Biochemistry **57** 4629-4637. Copyright 2018 American Chemical Society.) shows the simultaneous X-ray crystallography and spectroscopy method in more detail. A detector downstream collects the diffraction pattern from the crystals, which can then be used for determining the structure of the protein. The structure of PSII determined in this manner is shown in the figure. At right angles to the incident beam is the energy-dispersive emission spectrometer, which collects the entire emission spectrum from each X-ray laser shot, which is then averaged over many shots to improve the signal-to-noise ratio. The emission spectrum gives information about the electronic structure of the metal catalytic site, and the intactness and advancement of the catalyst through the enzymatic cycle as described in Figure 4.

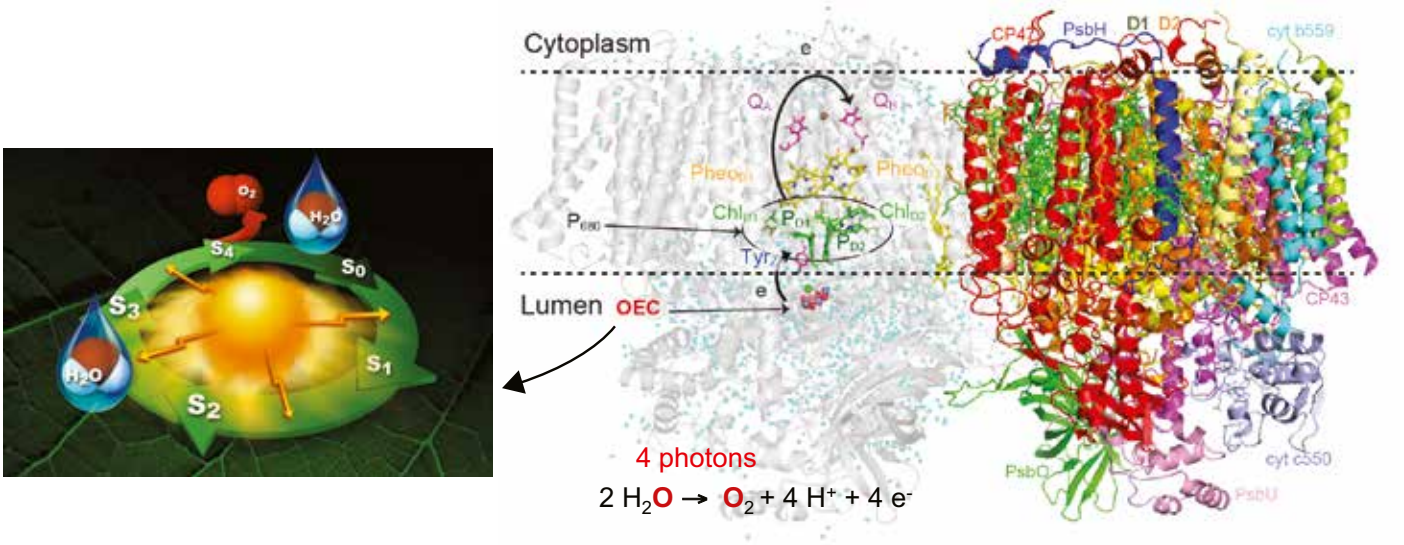

Figure 4. Kok cycle of the water oxidation reaction by Photosystem II. On the right is the detailed structure of the dimeric PSII protein determined using data from an XFEL. In the membrane portion are the helical structures, where PSII light absorption, charge separation and stabilization occur through a series of cofactors. The sequence shows the P_{680} reaction centre (a pair of chlorophylls) near the lumenal side, the series of chlorophyll and pheophytin molecules embedded in the helical part of PSII, and the acceptor quinone molecules on the cytoplasm side of PSII. Bound between the helical portion and three extrinsic peptides on the lumen side is the Mn_4CaO_5 cluster where the oxidation of H_2O to O_2 occurs. The hole created on the P_{680} reaction centre by light absorption and charge separation is filled by an electron from the Mn_4Ca cluster via a tyrosine residue. On the left is the schematic of the S-state Kok clock of intermediates for the water oxidation reaction. Four sequential photochemical events at the reaction centre leads to the extraction of four electrons (oxidation) from the Mn_4Ca catalyst resulting in the S-state intermediates (S_i, i=0 to 4). The formation of the transient S_4 state leads to the spontaneous evolution of O_2 and the Kok clock is reset to the most reduced S_0 state, and the enzymatic cycle begins again. (Graphic courtesy of Greg Stewart, SLAC.)

of these radicals and solvated electrons often leads to harmful reactions with proteins and lipids. To minimize such effects, and thereby prolong the time and dose a biological sample can be exposed to X-rays, X-ray crystallography and X-ray spectroscopy are usually carried out with samples in a frozen state, at cryogenic temperatures using liquid helium (approximately 4–10 K, –270°C) or liquid nitrogen (approx. 77 K, –200°C). Such low temperatures dramatically slow down the diffusion of radicals or solvated electrons, to increase the lifetime of samples and the X-ray dose they can be exposed to.

This, however, limits the usefulness of X-rays when we want to employ them as a tool to look at something 'physiological' and 'functional'. Here, we would need to obtain the information under more biologically relevant conditions, i.e., at room temperature, and conventional X-ray measurements are simply too damaging. The solution to this problem came from the most unexpected of places. When XFELs were originally proposed, most of the scientific community studying biological function were underwhelmed. How could these new ultra-bright X-ray sources, that deliver the same number of photons in a single ultrafast pulse that a synchrotron provides in a second, be useful for biology, when we already struggle to tame the damage caused by synchrotrons? Ironically, this very property, originally believed to sound the death knell of XFELs for biological studies, turned out to be its biggest virtue. While on longer timescales the accumulated dose determines the radiation damage (and not its rate), at extremely short timescales, the radiation damage can literally be outrun, and much higher X-ray doses can be used. XFELs provide ultra-short pulses of some femtosecond durations, which is too short a timescale (a femtosecond is 10^{15} of a second) for the diffusion of electrons or radicals. Using ultra-bright XFEL pulses that only last a few tens of femtoseconds, the sample is probed before the diffusion of radicals and solvated electrons has time to damage it. This probe-before-damage (or probe-before-destroy) concept is what scientists at XFEL facilities use to study many systems, including all biological ones. At the same time, each XFEL pulse is intense enough to provide enough X-ray diffraction and X-ray spectroscopy photons that, by adding many such snapshots, the desired information can be obtained.

Taking many snapshots to create movies to follow the reactions in real time

A high-resolution snapshot structure of any enzyme poised in a certain state reveals much about the system under study. Using the structure, one can learn and speculate about the reaction mechanism at play, especially

when combining it with other existing experimental information and results from theoretical models. While this is a good starting point for untangling how an enzyme functions, it may not be sufficient to solve the puzzle if, say, it was the equivalent of a jigsaw with too many missing pieces.

However, if one can obtain many snapshots of enzymes in action, at various moments during the reaction, the missing pieces begin to fall into place, removing the speculation. With the ultra-bright XFEL pulses, such an approach has become possible. Specifically, we use X-ray spectroscopy and X-ray crystallography with XFEL pulses to look at molecular structures of proteins while the reaction proceeds, after initiation with optical pump pulses. With a series of such snapshots at varying time delays, we can map out the changes that make the reaction proceed. The advantage of this approach is that one can look at the sequence of events step-by-step, from the structural point of view using crystallography, as well as from the chemical point of view, with spectroscopy that looks at the chemical states of metals. A series of time points will then help us understand how the reaction proceeds and why it happens in a certain way. Knowing this will likely give us important hints for understanding biochemical reactions, designing new bioengineering processes or mimicking the reactions by other means.

The photosynthetic water oxidation reaction

To illustrate the potential for this technique, consider our work on one of the most fundamental reactions in nature, the photosynthetic splitting of water and evolution of molecular oxygen by plants and cyanobacteria. We used the XFEL-based approach to study this photosynthetic water oxidation reaction, which occurs in a membrane protein, Photosystem II (PSII) (Figure 4). Plants, algae and cyanobacteria all use sunlight and water to fix carbon dioxide and make carbohydrates. The overall process involves a series of reactions carried out by many enzymes. However, the oxidation of water that occurs in PSII plays a

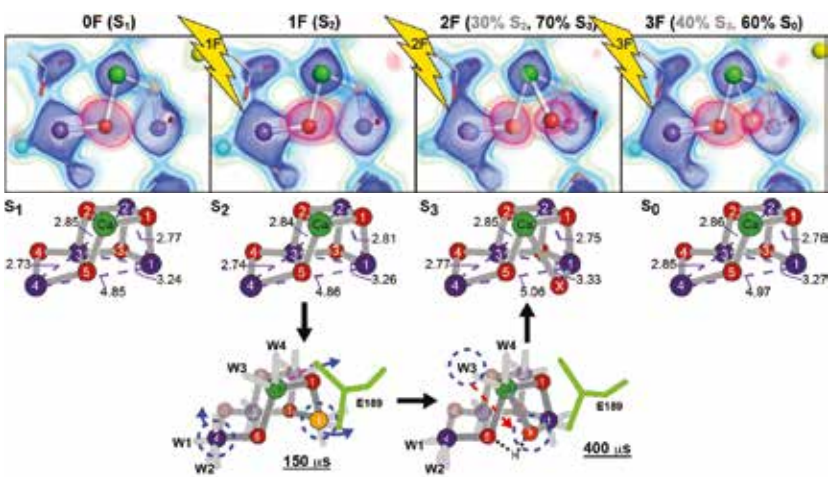

Figure 5. Where is the O–O bond formation site and how is the O–O bond formed? On top is a picture of a leaf with a proposed mechanism for which of the O atoms are involved in the O–O bond formation and O_2 formation (orange colour) in the Mn_4Ca cluster. The bottom panels (adapted from Kern et al., Nature, 2018) show how the data from XFEL crystallography is used to derive such detailed mechanisms. The electron densities and the structures of each of the S-states, advanced by visible laser excitation, and two time points at 150 µs and 400 µs between the S_2 and S_3 states was determined using the XFEL method described in this article. The electron densities identify where the Mn and the ligand atoms of Mn and therefore the structure of the cluster, and other residues are located and the inter-atom distances can be determined (purple – Mn, orange – O, green – Ca). The structures and, more importantly, the changes seen between the S-state transitions show which of the O atoms are candidates for the ultimate step involving the O–O bond formation and O_2 release. We clearly see the insertion of an O atom between Mn1 and Ca in the advancement between S_2 and S_3 states. The structures also show the role of Ca and indicate which water molecules could be involved in the reaction. This figure illustrates the power of the XFEL method for not only deriving the structures of proteins, but the dynamics of the catalytic reaction that informs about the intricacies of atomic motions involved in orchestrating catalysis.

crucial role, with water as the ultimate donor of electrons via splitting of the water into protons, electrons and molecular dioxygen. Protons and electrons are used as the energy source to drive the rest of the photosynthetic reactions, and oxygen is used for maintaining the life of organisms, including humans.

How does PSII carry out the water oxidation reaction? Photosynthetic systems have evolved an ingenious catalytic metal centre, called the Oxygen Evolving Complex (OEC), that consists of a Mn_4CaO_5 cluster, which catalyses the reaction, $2H_2O \rightarrow O_2 + 4e^- + 4H^+$ using the energy from 4 photons of light absorbed by the chlorophyll and other light-absorbing pigments. PSII carries out this reaction by coupling the 4-electron oxidation of water at the Mn_4CaO_5 cluster, with the 1-electron photochemistry occurring at the reaction centre, chlorophyll (Figure 4). The OEC cycles through 4 stable intermediate S-states (S_0 to S_3), where the progression through each of the states involves the abstraction of electrons and protons. Once 4 electrons are removed and 4 oxidizing equivalents are accumulated in the cluster (hypothetical S_4 state), similar to a charged capacitor, a spontaneous reaction occurs, with the release of O_2, and the cluster returns to the most reduced S_0-state.

We captured each intermediate state structure (S_0, S_1, S_2 and S_3) during this water oxidation reaction and observed how the structure of the OEC changes (Figure 5), together with the protein environment and water arrangement. We also determined the oxidation state of Mn for each snapshot, from XES data collected simultaneously with crystallography. This wealth of data from the snapshots is leading to an understanding of the mechanism for splitting of water to form dioxygen. We still do not know exactly which 2 oxygen atoms come together to make the O–O bond, nor how the 2 substrate water molecules come into the OEC from bulk water through the channels in the protein. Therefore, we would like to take more snapshots between the stable S-states to understand how one state advances to another and why one way is preferred but not another.

In addition, we are also considering why calcium is required in the cluster and what role it fulfils. Through the snapshot study, we now think that calcium may play an important role in shuttling substrate water molecules. Its ligation environment changes during the reaction, which was not previously expected.

Moving forward

At the time of writing, five XFEL facilities have become operational around the world, and additional facilities and major upgrades are underway. This will help to overcome current severe capacity limitations of these unique research tools and further establish methods using intense ultrafast XFEL pulses. Additional upgrades to new XFELs with much higher repetition rates will make it possible to create more complete sequences of snapshots and reveal even subtler changes. Both the increased capacity and enhanced performance of XFELs will help to perform experiments that reveal the inner workings of biological and chemical systems at room temperature and with atomic resolution, more accessible as an established method. We believe that the approaches described in this article can, in the near future, be applied and extended to study a wide range of enzymes and other systems. While most experiments currently use a light trigger to start the catalytic reaction, work is underway to create other time triggers, including rapid mixing schemes. As we make progress in overcoming the technical challenges for such studies, we are convinced that ultrafast X-ray pulses at XFELS will reveal many hidden secrets of nature's fundamental biological functions. ■

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Further reading

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