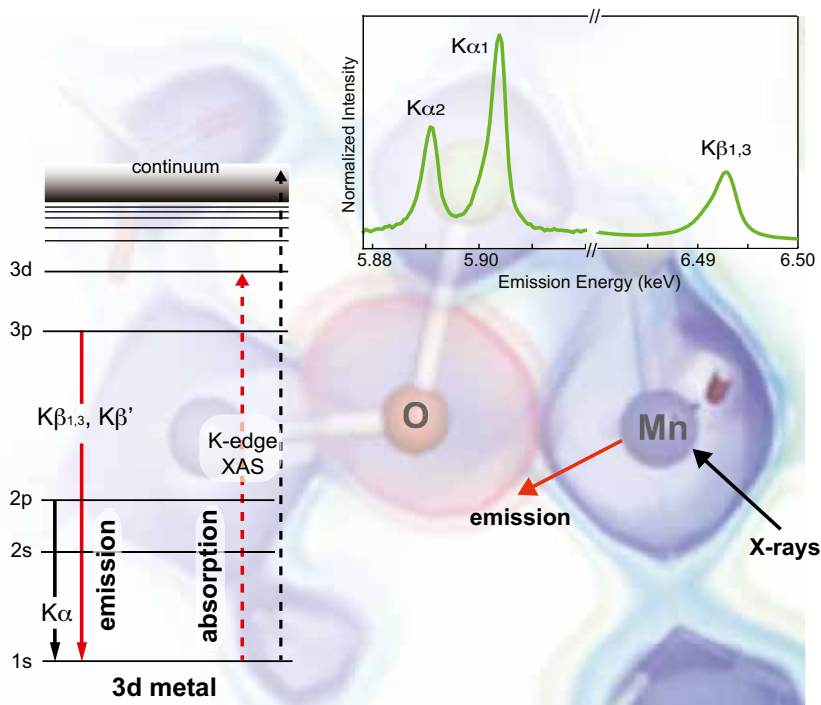


# How ultrafast X-ray pulses can reveal hidden secrets of photosynthesis

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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  $K\alpha$  and  $K\beta$  spectroscopy involves emission from 2p to 1s and 3p to 1s levels subsequent to the absorption of a photon from the 1s to the continuum. A typical  $K\alpha$  and  $K\beta$  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.

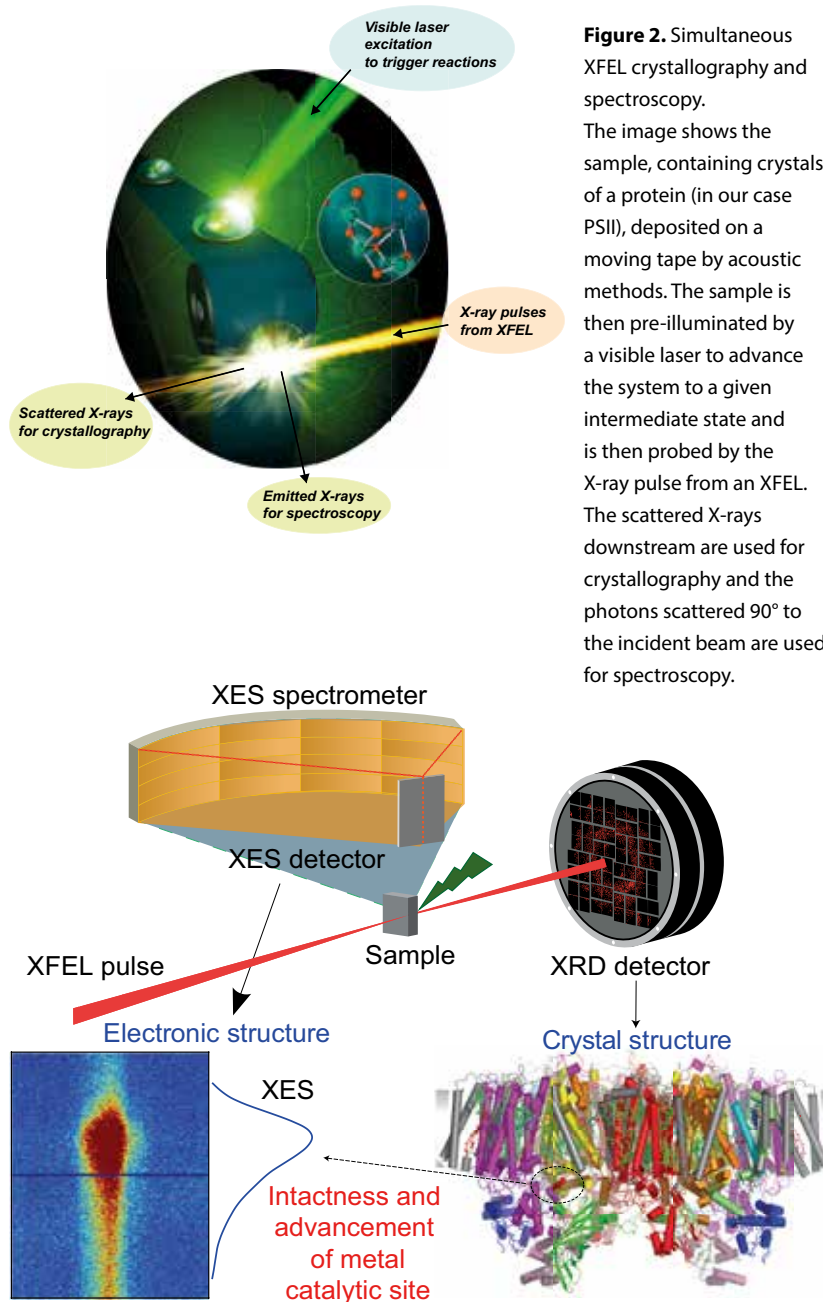
Can we combine these two powerful methods, X-ray crystallography and X-ray spectroscopy, for the study of metalloenzymes to simultaneously obtain the overall protein structure and the local electronic structure at the active centre (Figures 2 and 3)? Furthermore, we would ideally like to obtain this 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







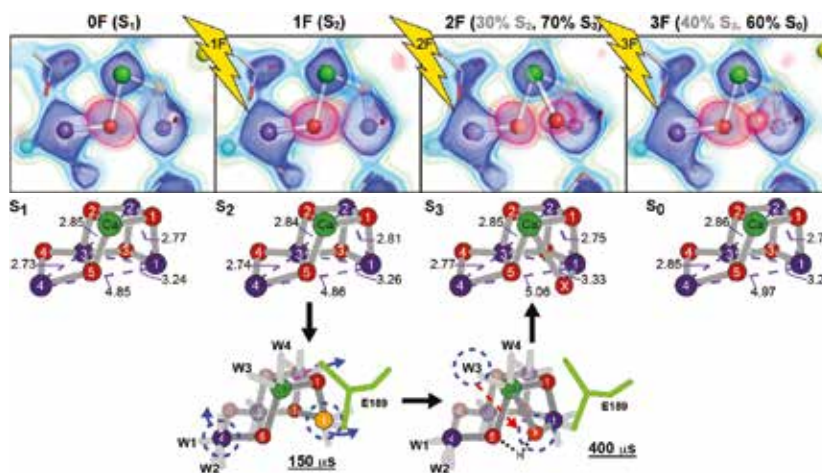
when combining it with other existing experimental information and results from theoretical models. While this is a good starting point for untangling how an enzyme functions, it may not be sufficient to solve the puzzle if, say, it was the equivalent of a jigsaw with too many missing pieces.

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<sub>2</sub> formation (orange colour) in the Mn<sub>4</sub>Ca 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<sub>2</sub> and S<sub>3</sub> 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<sub>2</sub> release. We clearly see the insertion of an O atom between Mn1 and Ca in the advancement between S<sub>2</sub> and S<sub>3</sub> 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  $\text{Mn}_4\text{CaO}_5$  cluster, which catalyses the reaction,  $2\text{H}_2\text{O} \rightarrow \text{O}_2 + 4\text{e}^- + 4\text{H}^+$  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  $\text{Mn}_4\text{CaO}_5$  cluster, with the 1-electron photochemistry occurring at the reaction centre, chlorophyll (Figure 4). The OEC cycles through 4 stable intermediate S-states ( $\text{S}_0$  to  $\text{S}_3$ ), 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  $\text{S}_4$  state), similar to a charged capacitor, a spontaneous reaction occurs, with the release of  $\text{O}_2$ , and the cluster returns to the most reduced  $\text{S}_0$ -state.

We captured each intermediate state structure ( $\text{S}_0$ ,  $\text{S}_1$ ,  $\text{S}_2$  and  $\text{S}_3$ ) 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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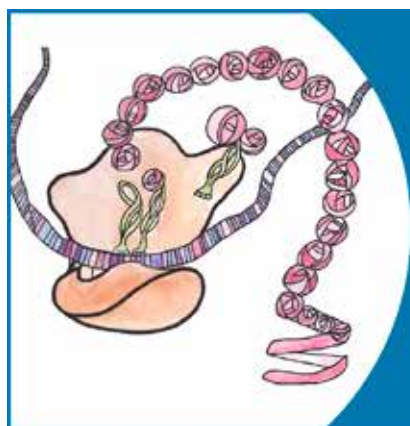
Vittal Yachandra received his BSc in Chemistry from Loyola College, University of Madras, and MSc in Chemistry from the Indian Institute of Technology at Kanpur, India. In 1975, he moved to the US and received his MS from the University of Chicago, and his PhD from Princeton University in 1982, working with Professor Thomas Spiro on resonance Raman spectroscopy of iron-sulfur proteins and X-ray spectroscopy of Zn and Co carbonic anhydrase. Photosynthetic water oxidation and the  $Mn_4Ca$  catalytic cluster became his research interests as a postdoctoral fellow working with Drs Melvin Klein and Kenneth Sauer at the Melvin Calvin Laboratory, Lawrence Berkeley National Laboratory (LBNL) at Berkeley in 1982, where he became a Staff Scientist in 1985. He is presently a Senior Scientist in the Molecular Biophysics and Integrated Bioimaging Division at LBNL. His research has focused on the use of EPR, X-ray spectroscopy and X-ray crystallography to study the  $Mn_4Ca$  water-oxidizing catalyst in photosynthesis and metalloenzymes, with special emphasis on the use of femtosecond X-ray laser facilities to study the time-evolution of the catalytic reactions, with the goal of applying the principles learned from nature to create/study bio-inspired systems. Email: [VKYachandra@lbl.gov](mailto:VKYachandra@lbl.gov)



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