Neutron crystallography sheds light onto the mechanisms of life

Biological macromolecules, like proteins and enzymes, play a crucial role in promoting and regulating the complex chemical processes that are the very basis of life. Understanding the structure of these molecules at an atomic level is the first step towards developing models of the processes keeping organisms alive. Prof John Helliwell (University of Manchester) is a pioneer since the 1970s of synchrotron X-ray light sources for protein crystallography. In an unexpected offshoot of using the polychromatic white beam of X-rays the analysis methods and software, in collaboration with Dr Marjorie Harding then of Liverpool University, were applied to neutron protein crystallography in the 1990s and 2000s. With colleagues in the neutron facilities in Grenoble and now also Lund there is major development and optimisation of neutron crystallography techniques as a powerful tool in structural chemistry and biology. This includes the study of the interaction of drugs with biological macromolecules. These methods and software are now also a workhorse at the USA's Oak Ridge National Laboratory neutron protein crystallography instruments. Prof John Helliwell has crystal structures in the Protein Data Bank spanning enzymes, lectins, crustacyanins and antibodies including complexes with sugars, drugs and imaging agents.

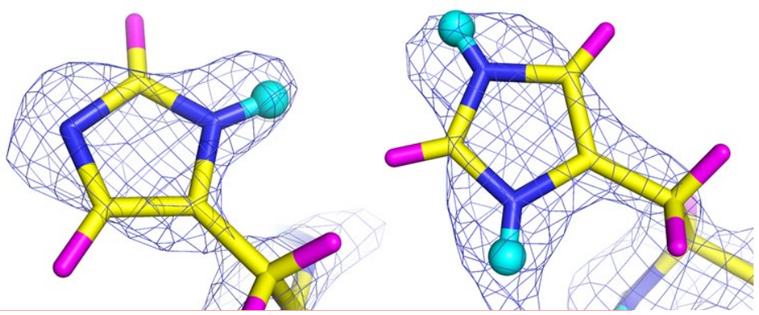
At a fundamental level, all chemical processes occurring in living organisms, from bacteria to complex multicellular organisms, are the result of an incredibly complex network of relatively simple chemical reactions, which create new molecules from the assembly of smaller molecular blocks or break down of larger molecules to generate and store chemical energy. The majority of these reactions do not occur spontaneously in nature, and living organisms had to evolve highly sophisticated methods to promote and carefully regulate those reactions that are necessary for their survival. A class of proteins known as *enzymes* act as the catalysts that can make otherwise unreactive species undergo the chemical transformations that keep organisms alive.

Enzymes are very large molecules typically comprised of thousands of atoms arranged in complex ways. Only a very small number of these atoms, the enzyme *active centre*, play a direct role in chemical reactivity, although the whole protein conformation forms to shape the active site of the chemical reactions and can influence the activity in subtle ways. For instance, small molecules can bind to receptor sites far away from the active centre and promote or inhibit the catalytic activity of the enzyme. Understanding how the structure and reactivity of an enzyme are related to each other and how the interaction with specific molecules, like natural molecules or artificial drugs, can be used to tune or inhibit the enzyme activity, and other biological functions at the molecular level. The structural studies can be used for developing efficient therapeutic approaches to wide classes of diseases and infections.

SEEING THE ATOMS IN ENZYMES: X-RAYS

The start of the determination of the atomic structure of enzymes and other biological macromolecules is mostly based on X-ray crystallography. This technique commenced in the early twentieth century with simple structures like sodium chloride. It progressively improved to ever more complex structures and synchrotron radiation crystallography now dominates (~90%) the Protein Data Bank depositions. They have also reached a high level of precision and reliability. The method exploits the interaction of X-rays with the electrons of the atoms of a sample. For crystallisation a biological molecule like an enzyme has to be prepared in pure form. An enzyme crystal is then exposed to an X-ray beam.

The exquisite sensitivity of neutrons as probe for determining the protonation states of histidine, a frequent player in enzyme mechanisms. Left singly protonated; right doubly protonated histidine in concanavalin A.



Measuring diffracted X-rays from the crystal yields the nature and position of the atoms within the crystal.

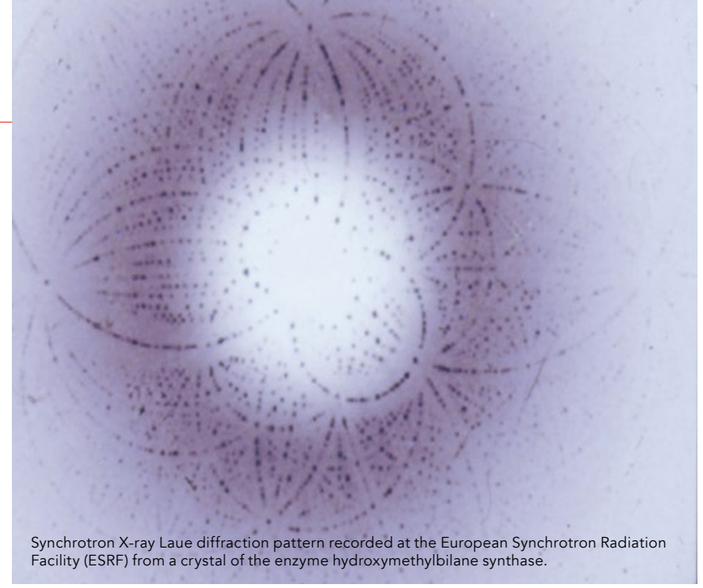
NEUTRONS IN MACROMOLECULAR CRYSTALLOGRAPHY

Despite their enormous success, X-ray diffraction techniques suffer from two important limitations. First, they interact weakly with the hydrogen atoms in a crystal. This is a major shortcoming, since the ionisable amino acids (aspartic or glutamic acid, histidine or lysine) are often involved in chemical reactions involving proton transfer and/or critical hydrogen bonding with ligands and bound water is involved in biological function or drug molecule interactions. In some situations, complementary techniques, like electron microscopy (cryoEM) and nuclear magnetic resonance (NMR), can be used to aid the identification of the hydrogen atoms. CryoEM and NMR are also essential when crystallisation fails or is poor. A second issue for X-rays, as well as electrons as probe, is that being ionizing radiation there is a progressive degradation of a crystalline sample during measurements. X-ray, or electron, induced radiation damage causes structural artefacts.

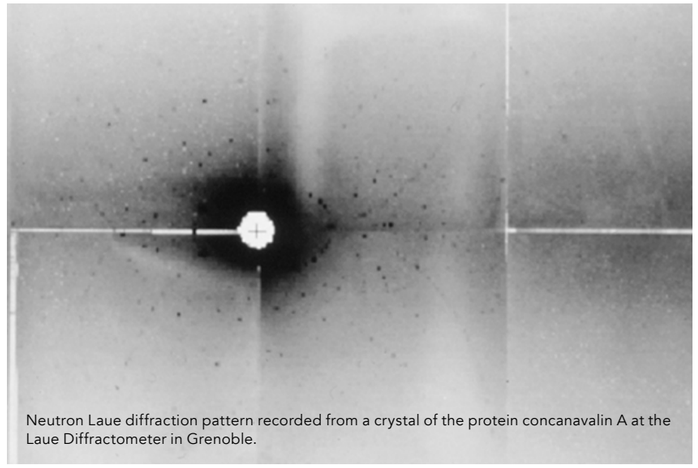
As Prof Helliwell explains, "Vital research breakthroughs in life sciences are being made in the areas of fundamental, industrial or structure-assisted drug design through the use of neutron macromolecular crystallography to achieve biomolecular structures with complete sets of atoms (including hydrogens) at unprecedented level of detail, at physiological relevant temperatures, and radiation damage free. The core need is where other probes fail and the understanding of a biological mechanism has to decide between equally plausible structural possibilities. The total number of neutron protein crystal structures is hundreds but each has served to resolve these structural hypotheses."

The work of Prof Helliwell has been the synergistic development of neutron macromolecular crystallography as a powerful complement to his synchrotron X-ray diffraction work.

How do neutrons interact with matter? Unlike X-rays, neutrons interact with the atomic nuclei in a crystal, rather than with the electrons. They can therefore be used to work out the positions of all the atoms present in a sample, including the hydrogen atoms usually replaced with deuterium. This isotopic substitution increases the signal-to-noise ratio, and it can provide a means to locate very accurately the positions of the hydrogen atoms. This substitution affects kinetics of reactions but not the structure. Furthermore, neutron measurements are unaffected by radiation damage effects, which paves the way for very high levels of accuracy in structural resolution. In physics accuracy and precision are not the same. X-ray and neutron crystal structures are both precise as the molecular models match the respective measurements. But taken together they constitute accurate molecular models. Neutron macromolecular crystallography has therefore already addressed some of the structural and mechanistic questions that have so far eluded X-ray, NMR and electron microscopy based research work and is a great potential for the future.



Synchrotron X-ray Laue diffraction pattern recorded at the European Synchrotron Radiation Facility (ESRF) from a crystal of the enzyme hydroxymethylbilane synthase.



Neutron Laue diffraction pattern recorded from a crystal of the protein concanavalin A at the Laue Diffractometer in Grenoble.

The work of Prof Helliwell has been instrumental in establishing neutron diffraction in macromolecular crystallography as a powerful complement to X-ray diffraction.

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INNOVATIVE INSTRUMENTATION AND METHOD BREAKTHROUGHS

However a great limitation of neutron sources is their relatively weak intensity of the beam flux, of the order of laboratory X-ray sources. It is therefore very important to optimise how the source is used as well as the instrumentation used to detect the scattered neutrons. Furthermore, it has proved greatly synergistic to apply the synchrotron white beam analysis methods to neutron crystallography data. For a long time, through the 1980s to 1990s, the scope of neutron macromolecular experiments had been drastically limited by the very large crystal volumes required in the measurements, which are difficult to achieve for most important and interesting proteins. Prof Helliwell has collaborated with neutron staff at the Institut Laue-Langevin (ILL) in Grenoble from the mid 1980s. This has led to greatly reducing the measuring times in these neutron experiments from a year to less than two weeks. Work by his former ILL PhD student Dr Blakeley has achieved a great increase of the molecular weight of the proteins studied, while at the same time dramatically reducing the crystal volumes requirements. These efforts were then greatly assisted by a local protein deuteration facility established by Dr Forsyth. Thanks to all these developments, sub-mm^3 crystals and proteins with large crystal cells (> 150 Å) can now be studied routinely and the data collection process has been reduced to a few days or, in some cases, even hours.

The expertise developed at Grenoble is being shared with the European Spallation Source (ESS) in Lund (Sweden) through Prof Helliwell's involvement as a Chair of the Science and Technical Advisory Panel (STAP) and his collaboration with Dr Matthew Blakeley (Institut Laue-Langevin). Next-generation instrumentation for crystallography with enhanced flexibility in the configuration of the detectors is being developed in Lund, under the leadership of Dr Esko Oksanen, which will extend the global capabilities of neutron crystallography to new frontiers. These technical developments also benefit from collaborations with CERN in Geneva, with their new detectors. It is nice to reminisce that this commenced from UK's Synchrotron Radiation Source (SRS) in the 1980s and an initiative at the Cornell University synchrotron source led by Prof Moffat who Prof Helliwell also worked closely with (full details are here:- https://zenodo.org/record/4381992#.YBAGKrPgrtQ) .

A NEW PERSPECTIVE ON ENZYMATIC REACTIONS

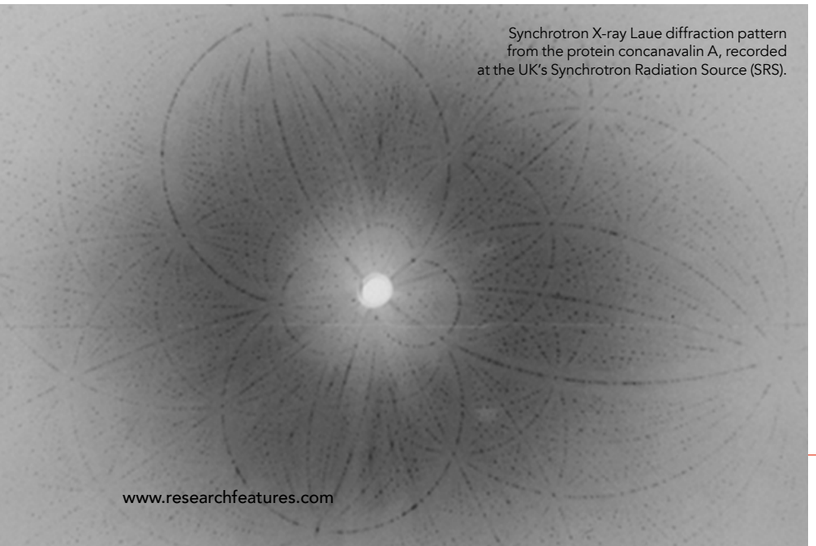
These world leading capabilities in neutron macromolecular crystallography have made it possible to address questions concerning the details of biological reactions that were unthinkable before this technique reached maturity. The methods allow the direct visualisation of the protonation state of reactive intermediates within enzymatic cycles, that is of how the binding of hydrogen atoms at specific sites promotes the catalytic activity of an enzyme. In 2014, neutron macromolecular crystallography was used, for instance, to unveil the structure of an important transient intermediate of the iron-containing enzyme cytochrome c peroxidase published in Science led by Professors Peter Moody, Emma Raven and Dr Blakeley.

We can now start to address questions concerning the details of biological reactions that were unthinkable before this technique reached it maturity.

This work resolved a long-standing question concerning the structure of this enzyme by conclusively establishing the involvement of an unprotonated ferryl ion (containing an electron deficient iron atom bound to an oxygen atom) as an active enzymatic intermediate. This finding has important implications for our understanding of the role of intermediates in iron-containing enzymes, which play a fundamental role in a number of biological processes including aerobic respiration, that is the cleavage of atmospheric oxygen molecules to produce chemical energy *in vivo*.

In another theme, commencing in 2013, neutron macromolecular crystallography was used to study the HIV-1 protease in complex with amprenavir, and then in 2016 the transfer of protons. The HIV-1 protease is a key drug target for HIV/AIDS therapy. Studying the structure and function of this enzyme and how hydrogen atoms move in response to changes in the enzyme environment, provides vital clues for understanding its resistance to drugs and for guiding the design of new drugs. By comparing the hydrogen atom distribution before and after the interaction of HIV-1 protease with the clinical drug darunavir, important insight was reached on how the acidity of the water medium surrounding the enzyme plays a key role in driving the simultaneous transfer of two protons between the drug and the active site of the enzyme.

These two examples showcase the reality and extraordinary potential of neutron macromolecular crystallography in the life sciences as a now indispensable tool for unlocking the structural and mechanistic details of large classes of important biochemical processes.



Synchrotron X-ray Laue diffraction pattern from the protein concanavalin A, recorded at the UK's Synchrotron Radiation Source (SRS).

Behind the Research

Professor John Helliwell

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Research Objectives

Professor Helliwell is currently engaged in a number of research crystallography projects in collaboration with University of the Free State, South Africa and with Imperial College London as well as with colleagues at the University of Manchester.

Detail

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Bio

John Richard Helliwell is a Fellow of the American Crystallographic Association, an Honorary Member of the British Biophysical Society, an Honorary Member of the British Crystallographic Association and a Corresponding member of the Royal Academy of Sciences & Arts of Barcelona, Spain. He is an Honorary Member of the National Institute of Chemistry, Slovenia.

He is Emeritus Professor of Chemistry at the University of Manchester and holds a DSc degree in Physics from the University of York.

Collaborators

- Dr Matthew Blakeley, LADI Instruments Leader of the Institut Laue Langevin;
- Dr Esko Oksanen, ESS's NMX Instrument Leader;
- Prof Trevor Forsyth, Group Leader at the Institut Laue Langevin and PI for DLAB Grenoble;
- Dr Zoe Fisher, Group leader for the Deuteration and Macromolecular Crystallization (DEMAX) user support laboratory at ESS.

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Personal Response

Neutron macromolecular crystallography has now reached a level of maturity sufficient for it to become a standard tool in fundamental science as well as in applied fields, like medicine and drug discovery. What are some of the currently unsolved questions in the life sciences that this technique will help to address, and what are the main challenges ahead for its widespread application?

" There are major new developments at both the Institut Laue Langevin and the upcoming European Spallation Source in a next wave of instruments. The range of biological structure and function developments that can now be analysed spans the following areas: enzyme catalysis involving proton transfer chemical reactions, ligand binding for drug discovery with computational modellers able to have complete structures with protonation and hydrogenation states complete, free of structural artefacts and at physiological temperatures. The method is being applied to the coronavirus main protease drug development (see https://coviddemystified.com/a-crystal-clear-view-of-the-sars-cov-2-protease/). "