A beginner's guide to neutron macromolecular crystallography

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Hydrogen atoms drive biological structure and function, but the lightest element is often unseen in three-dimensional macromolecule structures, hampering our understanding of biochemical processes. This guide will i) present how neutron crystallography uniquely reveals the experimental positions of hydrogen atoms and resolves mechanical controversies, ii) briefly introduce beamlines at neutron facilities, iii) discuss sample requirements and preparation and iv) familiarize the reader with neutron data and refinement statistics.

Introduction

The first three-dimensional structure of a protein was determined by Max Perutz and his colleagues in 1960 using X-ray crystallography. X-ray crystallography has transformed our understanding of biochemistry, including discoveries that have led to Nobel prizes being awarded in 1962 to Perutz and his colleague John Kendrew for the structural studies of globular proteins, to Dorothy Hodgkin in 1964 for the structures of vitamin B12, to Venki Ramakrishnan, Tom Steitz and Ada Yonath in 2009 for the structure of the ribosome and to Robert Lefkowitz and Brian Kobilka in 2012 for the structure of G-protein coupled receptors. To this day, X-ray diffraction remains the prevailing technique to determine the three-dimensional arrangement of carbon, nitrogen, oxygen, sulphur and phosphorous atoms in biological macromolecules, with ~90% of the biological structures deposited in the Protein Data Bank solved by X-ray crystallography.

While the performance of X-ray diffraction remains unmatched, X-ray structures have two shortcomings. First, hydrogen atoms are nearly always invisible and their positions and presence must be extrapolated from chemical knowledge. Second, X-rays damage biological molecules. Structural artefacts such as breakage of disulphide bonds, decarboxylation of glutamates and aspartates or reduction of metal centres may result from X-ray radiation–induced damages. Knowing the position of hydrogen atoms is not essential for projects that aim to determine the overall three-dimensional structure of proteins, DNA, RNA or complexes of these. However, this knowledge becomes essential in the investigation of enzyme catalytic mechanisms or of the hydrogen bond networks that govern ligand (e.g. pharmaceutical drug) binding. Similarly, radiation damage is not always an obstacle to structure analysis, unless the protein being studied contains X-ray–sensitive metal centres or redox cofactors. Neutron macromolecular crystallography is particularly suited to these projects seeking to experimentally locate hydrogen atoms or where radiation damage is a concern.

General overview

Neutron crystallography in a nutshell

The fundamental concepts of neutron crystallography are those of X-ray crystallography. A crystal of a pure biomolecule is exposed to an incident neutron beam (the incident neutron beam is the beam of neutrons that hits the crystal). The atoms in the crystal scatter the neutrons. The resulting diffraction pattern (determined by the crystal parameters and the crystal orientations in the beam) is collected on a detector and the position and intensities of the diffracted beams are measured. The main difference is that electron clouds scatter X-rays, whereas nuclei scatter neutrons. As with X-ray crystallography, the missing phase information must be recovered from additional crystallographic measurements or calculated (this is known as the 'phase problem'). In neutron crystallography, the phases can be calculated from a previously determined X-ray structure of the same macromolecule under the same experimental conditions (crystal system and temperature). Amplitudes (calculated as square root of the measured intensities) and phases are used to calculate three-dimensional neutron scattering length density (SLD) maps using the mathematical operation known as Fourier Transform. A model containing all atoms, including the hydrogens, is then built and refined by the experimentalist to best fit the experimental maps (Figure 1).

Because neutron crystallography uses the scattering from nuclei, hydrogen and its isotope deuterium are much more readily visualized than with X-rays. The experimental determination of hydrogen/deuterium positions gives direct experimental insight into chemical reactions. Furthermore, neutrons are a non-destructive

Summary Box

- Neutron crystallography resolves mechanistic questions by revealing the position of hydrogen atoms in biological macromolecules.
- Neutron crystallography is ideal to study the mechanism of redox enzymes because neutrons do not induce any measurable radiation damage in biological macromolecules.
- Neutrons are a non-destructive probe allowing neutron crystallographic data to be measured at physiologically relevant temperatures.

probe (the energy of the neutrons used is well below the energy needed to ionize the atoms, so there is effectively no radiation damage). The question then is: given the potential of neutron crystallography, why are less than 1% of the structures deposited in the Protein Data Bank solved by neutron crystallography? Part of the answer is that, as discussed above, neutron crystallography is best used for tackling very specific questions, such as gaining insight and resolving controversies in protein chemistry from crystallographic data. But there is also a major experimental hurdle to solving neutron structures: growing crystals large enough to compensate for the relative low flux of neutron beams available at any current neutron facilities. Hence, the success in determining a neutron structure relies almost entirely on the ability to obtain large crystals.

Instrumentation considerations

X-ray beamlines at synchrotron facilities are almost always monochromatic, meaning that optics are being used to irradiate crystals with incident X-rays at a single wavelength. Neutron beams produced at neutron reactors and neutron spallation sources have fluxes that are several orders of magnitude lower than those of X-ray beams (neutron spallation sources produce neutrons by bombarding high-energy protons onto a mercury or tungsten target; when a proton hits a mercury or tungsten atom, 20–30 neutrons are ejected or 'spalled'). Optics that allow selection of a spread of wavelengths are, therefore, used on most neutron diffractometers dedicated to biological crystallography, to increase the flux of neutrons delivered at the sample and simultaneously stimulate thousands of diffraction spots. This configuration is referred to as quasi-Laue geometry (Laue geometry describes a configuration that uses the entire neutron spectrum). In addition to this

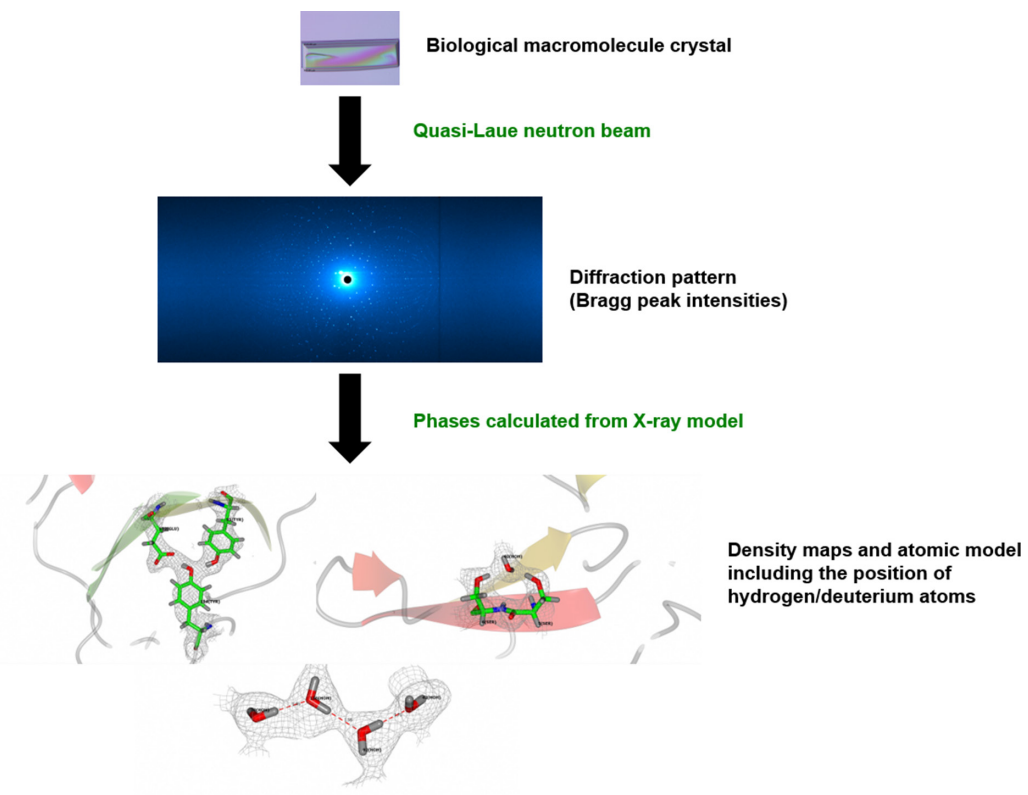

Figure 1. Neutron structure determination flowchart. The LPMO crystal displayed is 2 mm long and 0.5 mm wide. The dense diffraction pattern is typical of quasi-Laue crystallography. The grey mesh is the three-dimensional neutron SLD map in which carbon, oxygen, nitrogen and deuterium atoms are modelled. (Images courtesy of W. B. O'Dell.)

different polychromatic beam, the neutrons used for macromolecular crystallography are typically in the 2–5 Å range, compared to ~1 Å X-ray beam. This is because the diffracted intensity is proportional to the square of the incident wavelength, and is therefore stronger at longer wavelengths – this consideration is important in a flux-limited technique. In addition, the longer wavelengths allow dense quasi-Laue diffraction patterns to spread out because the diffraction angle increases with the incident wavelength according to Bragg's Law ($2d \sin\Theta = n\lambda$, where d is the interplanar spacing in the crystal, Θ is the angle of diffraction and λ is the incident wavelength). The larger angles lead to a detector geometry which is also very different from a detector installed on an X-ray beamline. Detectors on neutron beamlines are typically large and fixed, and they surround the crystal position to collect all the Bragg spots that are simultaneously stimulated by the quasi-Laue beam.

Sample considerations

Deuterium labelling. Fully or partially deuterated crystals are used because deuteration increases the signal over noise ratio of the data and enhances the visibility of hydrogen positions in the resulting neutron SLD maps. This section explains why.

Unlike X-rays where scattering is proportional to the number of electrons, neutrons interact with atomic nuclei and there is no linear relationship between an element's atomic number and its neutron scattering cross-section. Furthermore, because neutrons interact with nuclei, isotopes of the same element interact with neutrons differently. The neutron scattering cross-section of an element includes both a coherent component and an incoherent component (Figure 2). Coherent scattering is directional and gives rise to Bragg spots. Incoherent scattering is uniform in all directions and contributes to background. Both types of scattering occur upon the interactions of neutron beam with the sample.

The coherent component contributes to the intensity of the Bragg reflections, while the incoherent component contributes to the background noise in the diffraction image. The coherent and incoherent cross-sections of deuterium make it a more suitable isotope to measure diffraction data from biological molecules. Hydrogen scatters neutrons with about half the magnitude of the other nuclei found in biological macromolecules. In addition, the coherent interaction between neutrons and hydrogen nuclei leads to a phase shift of π in the diffracted neutron radiation. This additional phase shift results in a negative coherent scattering length, which hampers the interpretation of the resulting neutron SLD maps at typical resolutions (2.0–2.5 Å) because of cancellation effects. In contrast, the scattering length of deuterium is of the same sign and of the same magnitude as that of other nuclei found in biological macromolecules. Furthermore, the incoherent scattering of deuterium is 40 times lower than that of hydrogen atoms. As some half of the total atoms in a biological macromolecule are hydrogen and biomolecule crystals contain 50% solvent in average, fully or partially deuterated crystals produce stronger diffraction patterns, with much reduced background and increased signal, and the visibility of hydrogen atom positions is enhanced.

Crystals can be either fully deuterated or partially deuterated. In the former case, the biological molecules would be produced in its deuterium-labelled form (e.g. micro-organisms grown in deuterated culture medium) and the crystals grown from D_2O-prepared crystallization solutions. In the latter case, the biological molecule is produced in its hydrogenated form and the crystals are labelled at a later preparation stage. This deuterium labelling of crystals results in a complete exchange of the light (H_2O) water molecules in the crystal to heavy (D_2O) water molecules and to the exchange of the hydrogen atoms bound to oxygen and nitrogen, in the biological molecule or its ligands, to deuterium atoms, or about 25% of the total number of hydrogen atoms.

Visibility – or lack thereof – of metal ions and ligands. As many metal atoms have relatively small coherent neutron scattering lengths, the 'neutron maps' often fail to show these metal ions clearly at typical resolutions (2.0–2.5 Å). In order to confirm the position of a metal ion, it is important to collect a complementary X-ray data set, ideally on the same crystal (or a crystal grown under identical conditions) and at the same temperature. The electron density maps

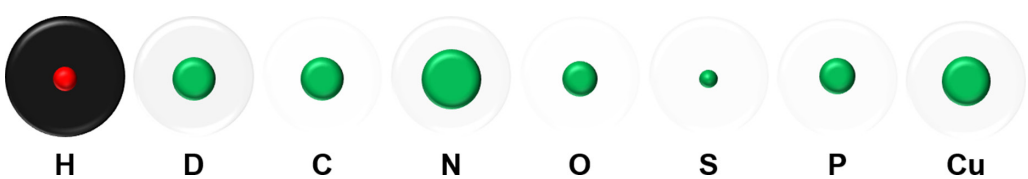

Figure 2. Incoherent neutron scattering cross-sections and coherent neutron scattering lengths for selected elements. Relative incoherent scattering cross-sections are represented by the disc coloured on a grey scale (dark: high incoherent cross-section, light: low incoherent cross-section), and relative coherent scattering lengths are represented by the red and green discs. The red disc for hydrogen indicates the negative sign of its scattering length, while those shown in green are positive.

can be superimposed onto the neutron scattering density maps to confirm the presence and position of a metal ion. A similar approach is often used to model ligands rich in hydrogen atoms, as the presence of hydrogen may lead to cancellation of the neutron scattering length density for that ligand. Ideally, such a ligand would be synthesized as its deuterated form, but if such labelling is challenging (or price prohibitive), then an X-ray data set will be needed to reveal the position of the ligand.

Large crystal growth. Large crystals are required to produce measurable diffraction at high enough resolution to solve the biochemical questions being asked. (Deuterium atoms can be unambiguously modelled from data extending to 2.5 Å resolution or higher.) The growth of large crystals requires crystallization set up with protein volumes ranging from tens to hundreds of microlitres. Sitting drops or batch methods are best suited to produce crystals of suitable volumes. A combination of micro- and macro-seeding, where a crystal is transferred to a fresh protein solution in the metastable state, allowing further growth, can also be used.

Data considerations

Data collection. Neutrons are a non-destructive probe. Therefore, most data sets are collected at room temperature from crystals mounted in quartz capillaries. The capillary also contains mother liquor prepared in D_2O to prevent crystal dehydration during data collection. Data collection at cryogenic temperature using a nitrogen cryostream is possible and necessary to capture unstable intermediates.

Data processing and statistics. Neutron diffraction data are reduced in a similar way to X-ray data. Diffraction patterns are indexed, integrated and scaled to produce a set of merged reflections. A major difference arises from the use of a polychromatic incident radiation (quasi-Laue geometry). The data must be wavelength normalized to account for the shape of the incident neutron spectrum.

The completeness of neutron data sets is often lower than that of their X-ray equivalent. The geometry of the quasi-Laue method and of the detector used (e.g. cylindrical image plate detectors lack detection in the direction perpendicular to the neutron beam) is in part responsible for this. In addition, neutron data collection typically takes days or even weeks. A project may be allocated only a fixed number of days, which may prevent the collection of a 100% complete data set.

Structure refinement. Structure refinement can be performed against neutron data alone or both neutron and X-ray data collected from the same crystal at the same temperature. The X-ray terms dominate to locate the heavier atoms and the neutron terms for the lighter atoms. Software packages such as the *phenix.refine* suite have been developed to support the refinement of neutron data. The process of refining a neutron model parallels the refinement of an X-ray model. Careful examination of the neutron SLD maps is performed to determine the protonation state of titratable residues such as histidine, aspartic and glutamic acids, lysine and tyrosine. Difference maps are used to further support the interpretation. The orientation of the water molecules can also be uniquely resolved from the neutron SLD maps.

Applications

References to our work and to the following examples in the area of redox chemistry are given in the *Further Reading* section.

In recent years, neutron crystallography has played a pivotal role in the studies of the enzymatic mechanism of several enzymes that catalyse redox chemistry. In these studies, critical knowledge is gained of the chemical nature of small molecules liganded to metal centres, e.g. H_2O, OH^-, H_3O^+ or O_2, H_2O_2, HO_2^- and O_2^{2-} are indistinguishable by X-ray crystallography, which only reveals the position of the oxygen atoms in these species. Similarly, the contribution of water molecules to proton transfer pathways can be elucidated, in addition to the experimental determination of the protonation states of key amino acids. It is also important to note that the redox state of the enzyme remains unperturbed during the diffraction experiment because neutrons are a non-destructive probe. Our laboratory has been using neutron crystallography to investigate the enzymatic mechanism of the recently discovered lytic polysaccharide monooxygenases (LPMOs). Understanding the catalytic mechanism of LPMOs and how to tune their functionality is central to efficient utilization of plant biomass.

The structure of copper-containing nitrite reductase (CuNIR) recently refined against neutron and X-ray crystallographic data revealed the protonation states and chemical nature of ligand species at the active site of CuNIR. The data support a hydroxide anion species (OH^-) liganded to the Cu ion and a doubly protonated catalytic histidine. The chemical features revealed by the neutron structure allowed the authors to reconcile divergences between previously published experimental results and DFT calculations.

Redox enzymes often rely on proton-coupled electron transfers (PCET) to carry out their chemistry. Neutron crystallography can reveal proton transfer pathways without perturbing the electronic state of the enzyme redox centre. The neutron structures of substrate-free ascorbate peroxidase and ascorbate-bound ascorbate peroxidase played an important role in resolving the role of active site residues and of water molecules in the transfer of proton at the active site of the enzyme. ■

Further reading

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