Neutron Protein Crystallography:

A Complementary Tool for Locating Hydrogens in Proteins

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Abstract

Neutron protein crystallography is a powerful tool for investigating protein chemistry because it directly locates hydrogen atom positions in a protein structure. The visibility of hydrogen and deuterium atoms arises from the strong interaction of neutrons with the nuclei of these isotopes. Positions can be unambiguously assigned from diffraction at resolutions typical of protein crystals. Neutrons have the additional benefit to structural biology of not inducing radiation damage in protein crystals. The same crystal could be measured multiple times for parametric studies. Here, we review the basic principles of neutron protein crystallography. The information that can be gained from a neutron structure is presented in balance with practical considerations. Methods to produce isotopically-substituted proteins and to grow large crystals are provided in the context of neutron structures reported in the literature. Available instruments for data collection and software for data processing and structure refinement are described along with technique-specific strategies including joint X-ray/neutron structure refinement. Examples are given to illustrate, ultimately, the unique scientific value of the neutron protein crystal structure.

Introduction

Proteins, and more specifically enzymes, are the lifeblood of cellular processes. Enzymes catalyze a broad array of chemical reactions essential to life. No less than 20,000 distinctive enzyme-catalyzed reactions are likely across all living organisms [1], controlling processes as diverse as the capture and conversion of energy from light, cell signaling, DNA translation and repair, and metabolic pathways. Most of these catalytic functions exhibit an exquisite chemical specificity, for both substrate and product, which is encoded in the precise three-dimensional organization of amino-acids at and leading to the active site of the enzyme. Enzyme activity is tightly orchestrated and controlled by a variety of post-translational modifications (phosphorylation, glycosylation, ubiquitination, acetylation, lipidation) and allosteric regulation

mechanisms. Local environment, pH, and chemical flux also trigger significant structural reorganization contributing to activity modulation [2-5].

Hydrogen atoms are central to enzyme chemistry, as ultimately, reaction rates and chemistry are dependent upon the coordinated changes in local electrostatics, hydrogen-bonding interactions, and protonation states of catalytic residues along the reaction coordinate. Therefore, understanding enzyme chemistry at the atomic level requires the visualization of hydrogen atoms on active site and remote residues, co-factors, substrate, and water molecules. This information can be challenging to obtain. While most structure-based knowledge arises from X-ray crystallography, hydrogen atoms are exceedingly difficult to visualize in X-ray structures. When data are available to ultra-high resolution, few hydrogen atoms may be visible or their positions may be inferred from precise geometrical parameter analyses [6]. The combination of atomic resolution X-ray crystallographic data with quantum chemistry or charge density analysis can then provide a further level of detail on the chemical profile of the enzyme [7-9]. However, even when such ultra-high resolution data can be obtained, a significant fraction (typically > 50%) of those more mobile or labile hydrogen atoms remain difficult to discern, leaving specific questions concerning catalytic mechanism unanswered.

Figure 1. **Incoherent neutron scattering cross sections and coherent neutron scattering lengths for selected elements.** Relative incoherent scattering cross sections are represented by the left hemispheres, and relative coherent scattering lengths are represented by the right hemispheres. The red hemisphere for hydrogen indicates the negative sign of its scattering length while the others shown in green are positive. Incoherent cross sections and coherent scattering lengths are not represented on the same scale.

The difficulty of locating hydrogen atoms using X-ray crystallography can be circumvented by neutron protein crystallography. This is because the coherent scattering lengths of hydrogen (H) and the hydrogen isotope deuterium (D) for neutrons are similar in magnitude to those of carbon, nitrogen and oxygen (Figure 1) [10]. A complication of neutron protein crystallography is that the scattering length of hydrogen is negative while that of carbon (C), nitrogen (N) and oxygen (O) are positive, which gives rise to density cancelation in Fourier maps that can hamper interpretation and analysis. In contrast, D has a neutron scattering length of the same sign as the heavier atoms and thus gives a clear positive peak in the nuclear density maps. While visibility of hydrogen atoms requires neutron crystallographic data at resolution of 2.0 Å

or better, deuterium atoms are readily visible, in crystallographic structures identical to their hydrogenated counterparts [11-15], at typical resolutions of 2.5 Å or better.

Despite a number of significant technical challenges that we will discuss in this review, the ability to experimentally locate deuterium makes neutron diffraction a method of choice for visualizing the positions of hydrogen in enzymes, whether used as a standalone technique or in complementary with Nuclear Magnetic Resonance (NMR) spectroscopy or high resolution X-ray crystallography. Neutron protein crystallography has, for example, been used to locate hydrogen atoms and determine the protonation states at the active site of HIV-1 protease (HIV Pr) [16, 17], beta-lactamase [18-20], dihydrofolate reductase (DHFR) [21, 22], carbonic anhydrase [23-26], xylose isomerase [27-33]. These studies have been essential in identifying active site residues with perturbed pK_a 's and contribute to the growing body of knowledge on pK_a modulation at the active site of enzymes.

In this review, we focus on practical considerations to help guide the successful development of neutron protein crystallography projects by newcomers. (The interested reader is encouraged to find more historical perspectives on NPC in the works by Niimura and Podjarny [34] and Blakeley [35].) A flow chart of the steps involved in neutron crystallographic structure determination is presented in Figure 2. We specifically review H/D exchange protocols, crystal growth methods, data collection strategies and structure refinement options that have been used for the 85 neutron protein structures currently deposited in the Protein Data Bank (October 1, 2015). We also give a perspective on the future of NPC illustrated with recently determined neutron protein structures.

Figure 2. **Neutron structure determination flow chart.** The flow chart shows the steps in neutron protein crystallography. Most steps are similar to those of x-ray protein crystallography. D labeling or H/D exchange and growth of large crystals are specific steps for the neutron experiment.

Hydrogen/Deuterium Isotopic Substitution

As stated previously, replacing H for D in a protein crystal provides significant benefits to the neutron protein crystallography experiment. The greater magnitude of the D coherent scattering length (6.671 fm) compared to H (-3.741 fm) results in a larger $\sum |\vec{F}|$ and, therefore, greater average reflection intensities. Simultaneously, the six-fold decrease in the D incoherent scattering length (3.99 fm) compared to H (25.27 fm) dramatically reduces the isotropic background intensity. The gain in signal and reduction in noise allow datasets to be collected from smaller crystals ($< 0.5 \text{ mm}^3$) or with reduced total exposure times (< 10 days) [13, 20, 36-38]. Furthermore, the D coherent scattering length is positive as is that of common atoms found in proteins while the coherent scattering length of H is negative (Figure 1). At the typical resolutions (1.9-2.3 Å) obtained for neutron protein crystallographic data, D substitution simplifies the analysis of neutron density maps by minimizing the number of sites where the negative coherent scattering length of H atoms leads to density cancellation. Figure 3 demonstrates the greater extent of significant neutron density in the $2F_o - F_c$ map from a fully D-labeled, or perdeuterated, crystal of beta-lactamase as compared with the map from an H/D-exchanged crystal. Both datasets were measured on the LADI III diffractometer at Institut Laue-Langevin and extend to 2.0 Å resolution [39]. However, data for perdeuterated beta lactamase were collected from a smaller crystal and in half the measurement time than was required for the H/D-exchanged protein [18, 19]. In the case of aldose reductase, perdeuteration produced an improvement in diffraction resolution from 4.5-2.0 Å that enabled neutron data collection and structure determination [13, 40, 41].

Figure 3. **Neutron density maps from H/D-exchanged and perdeuterated beta-lactamase.** Top: the $2F_o - F_c$ map from the H/D-exchanged crystal (PDB 2WYX) is shown for residue Arg 61. Bottom: the corresponding $2F_o - F_c$ map from the perdeuterated crystal (PDB 2XQZ) is shown. Both maps are contoured at 1.5σ . Structures were drawn using CCP4MG with coordinates taken from the PDB [42].

H/D exchange

Bulk solvent, ordered water molecules, and hydrogen atoms bound to protein oxygen, nitrogen, or acidic carbon atoms such as histidine C^{ε1} [43] [*i.e.*: titratable hydrogen atoms) can all be replaced with deuterium by preparing all reagents in deuterium oxide (D₂O) either during or after complete crystal growth. Of the neutron protein crystal structures currently deposited in the PDB (Supplementary Material Table S1 and www.rcsb.org), approximately half were determined from proteins with titratable hydrogen atoms exchanged during crystallization. In this approach, the protein sample is exchanged by dialysis [44] or by repeated concentration and dilution into a D₂O-prepared buffer solution. Similarly, crystallization salts and/or precipitants are dissolved in D₂O thus creating an H/D-exchanged mother liquor. The crystallization experiment is then setup in the same way as that performed with H₂O solutions. Re-optimization of crystallization conditions may be necessary for D₂O solutions due to the reduced solubility of proteins in D₂O and the differences in physical properties of H₂O and D₂O [45-47] (Table 1). The reduced solubility can be addressed by decreasing the crystallization agent concentration(s) [18, 19, 48], reducing the protein concentration [11, 14, 49], increasing the crystallization temperature [50, 51] or by varying these parameters simultaneously (see *Crystal Growth*).

Property	H ₂ O	D ₂ O
Bond energy, (kJ mol bond ⁻¹ , 0 K)	458.9 (H-O-H → •O• + 2H•)	466.4 (D-O-D → •O• + 2D•)
Boiling point (° C)	100.0	101.42
Density (kg m ⁻³ , 25° C)	997.05	1104.36
Dynamic viscosity (mPa s, 25 ° C)	0.8909	1.095
Melting point (° C)	0.00	3.82
Molar mass (g mol ⁻¹)	18.015265	20.027508
pK _w	13.995	14.870

Table 1. H₂O and D₂O physico-chemical properties.

For the remaining structures deposited in the PDB crystals were grown to final size in H₂O mother liquor and then subjected to H/D exchange either by vapor equilibration in capillary (Figure 4) or soaking in an artificial H/D-exchanged mother liquor.

Figure 4. **H/D exchange of a fully grown crystal in the capillary mount by vapor diffusion.**

(1) Crystals are drawn into the capillary from the crystallization setup. (2) Excess mother liquor is removed from the crystal (3) exposing the crystal surface for exchange. (4) Plugs of artificial mother liquor prepared in D₂O are added on each side of the crystal to drive the exchange in the closed environment provided by (5) the sealed capillary.

Exchange rates of individual hydrogen atoms in proteins in solution vary greatly depending upon factors including hydrogen bonding, solvent exclusion by protein folding, solvent pH, and incubation temperature [52-55]. The crystal lattice further limits diffusion of water to the network of pores formed by the packing of protein molecules [45]. These factors make the choice of H/D equilibration time arbitrary. In practice crystals are exchanged from the time the crystal is fully grown until data collection. Reported times for H/D exchange after crystallization range from seven days for diisopropyl fluorophosphatase [56, 57] to ten years for a crystal of myoglobin [58]. By analyzing ten neutron structures deposited in the PDB, Bennett *et al.* [59] concluded that H/D exchange by crystal soaking is likely to reach equilibrium after 30 days.

Two structures of HIV Pr allow for an interesting comparison of soaking and vapor diffusion exchange methods. Adachi *et al.* [16] determined a structure of HIV Pr (PDB 2ZYE) from a crystal that was soaked for 14 days in artificial D₂O mother liquor with pD = 5.0 (pD =

pH + 0.4) at 20 °C and observed approximately 54% exchange of backbone amide hydrogens. Weber *et al.* [17] also determined a structure of HIV Pr (PDB 4JEC) in which approximately 71% of the backbone amide hydrogens exchanged after vapor equilibration with D₂O solution with pD = 5.6 at 17 °C for 28 days. Remarkably, as shown in Figure 5, the patterns of backbone amide exchange in the two structures are quite distinct despite very close agreement in atomic positions (< 0.5 Å root mean square deviation). Multiple experimental distinctions may have contributed to these differences. The crystals were grown under significantly different crystallization conditions. The structures were also obtained with different small molecule inhibitors bound at the HIV Pr active site, and it is known that different modes of ligand binding can influence backbone amide exchange patterns [60]. In addition, the 2ZYE model was refined using phenix.refine [61] while the 4JEC model was refined using CNSsolve [62].

Figure 5. **Backbone amide H/D exchange observed in two neutron structures of HIV-1 protease.** Left: A ribbon diagram of the structure obtained after crystal soaking for 14 days is shown (PDB 2ZYE). Right: The corresponding diagram for the structure obtained after vapor diffusion H/D exchange for 28 days is shown (PDB 4JEC). In both structures, residues are colored by the percent of D substitution observed (color bar) in the refined model. Structures were drawn using UCSF Chimera with coordinates from the PDB [63].

Perdeuteration

Titratable hydrogen atoms represent approximately 25% of the total hydrogen atoms of any proteins. In order to exchange the remaining 75% attached to carbon atoms, proteins need to be perdeuterated during synthesis. Expression of perdeuterated protein in fully D-labeled growth media has yielded neutron protein crystal structures currently deposited in the PDB for six proteins, namely myoglobin [64, 65], aldose reductase [13, 41], transthyretin [38], type III antifreeze protein [66, 67], beta lactamase [19, 20] and rubredoxin [3, 36, 37, 68, 69].

Each perdeuterated protein was expressed recombinantly in the host *Escherichia coli*. Cultures must be progressively adapted to grow in minimal media formulated with D₂O, H/D-exchanged salts, and uniformly D-labeled carbon source prior to induction of expression either in shake flasks or in a bioreactor system. Methods for perdeuterated protein expression specifically for neutron protein crystallography have been described in detail by Meilleur *et al.* [44, 70] for cytochrome P40cam and Golden *et al.* [48] for cholesterol oxidase. These studies describe two

alternate approaches to growing of *E. coli* in fully deuterated medium in a bioreactor: the former used glycerol-limited fed-batch cultivation while the latter culture was grown until complete depletion of the culture broth initial carbon source content. Labeled proteins are generally isolated and purified using H₂O-formulated buffers due to the cost of D₂O and D-labeled reagents. The protein is then either exchanged back to D₂O buffer for crystallization or crystallized in H₂O conditions and H/D-exchanged as discussed above.

One structure of HIV Pr has been determined from 85% uniform D-labeled protein since it was observed that fully deuterated HIV Pr would not form crystals with sufficient volume for neutron diffraction [17]. Uniformly D-labeled protein can be expressed with $\leq 95\%$ D incorporation from *E. coli*, other prokaryotes, or even eukaryotic hosts using techniques largely developed for NMR experiments [71-74]. However, except for the case of HIV Pr, uniform partial deuterium labeling has not been applied in the field of neutron protein crystallography. Partial D-labeling does reduce incoherent background in the diffraction experiment, but it requires refinement of the H/D-occupancies for all sites in the model structure which considerably increases the number of parameters to refine [17] (see *Structure Refinement*). In addition, sites with H and D occupancies of approximately 60% and 40% respectively would also have null or insignificant density in Fourier maps due to the negative H and positive D coherent scattering lengths further complicating the structural analysis. More neutron diffraction experiments using crystals of partial uniform D-labeled protein will need to be conducted to fairly assess this approach.

Selective H-labeling in a perdeuterated background has been applied in neutron diffraction studies of rubredoxin (PDB 3KYY) [68, 69] and type III antifreeze protein (PDB 4NY6) [4, 75] with the ultimate goal of using neutron data to solve protein structures by direct methods [76]. Media supplementation with unlabeled α -ketoisovalerate during perdeuterated protein expression resulted in H-labeling of valine and leucine methyl groups producing (H- γ methyl)-valine and (H- δ methyl)-leucine uniformly throughout the proteins [77]. Fisher *et al.* [4] compared neutron diffraction datasets for perdeuterated and CH₃-H-labelled antifreeze protein to demonstrate the negative effects of protein H atoms on the observability of structured water molecules in neutron density maps. Theoretically specific H-labeling in a perdeuterated background can enable direct determination of model phases for structure solution; however, this application of neutron protein crystallography with selective labeling has yet to be demonstrated [76].

Crystal Growth

The diffracted intensity in Bragg reflections in a single crystal experiment can be written as:

$$\langle I \rangle \propto \frac{I_0 \times |F|^2 \times V \times \lambda^2}{v_0^2} \quad (1)$$

where $\langle I \rangle$, I_0 , $|F|$, V , λ , and v_0 are the diffracted intensity, incident beam intensity, structure factor magnitude, volume of the crystal, incident wavelength and volume of the unit cell, respectively [78]. Neutron beam fluxes are inherently many orders of magnitude weaker than synchrotron X-ray beams[79]. Therefore, neutron crystallography requires crystals that are at least three orders of magnitude larger than crystals suitable for X-ray crystallography because, as shown in Equation (1), the diffracted intensity is directly proportional to the incident beam intensity.

The neutron crystallography experiment typically follows X-ray crystallography experiments that, despite providing high-resolution diffraction data, fell short of unambiguously answering mechanistic questions due to the lack of information on hydrogen atom positions. All neutron crystallography projects have the starting advantage of known crystallization conditions, but these conditions most likely will need to be further optimized to sustain the growth of large crystals suitable for neutron data collection. Crystals typically need to be greater than 0.1 mm³ in volume (Table S1 and reference herein) and must be well ordered to diffract to sufficient resolution [80].

It is important to note that while the beam flux is the dominant requirement for large crystals, the volume of the unit cell is also a significant parameter. Equation (1) shows that the diffracted intensity is inversely proportional to the v_0^2 . In cases where several crystal forms can be grown, crystallization conditions promoting smaller unit cells should be favored. Growing large crystals requires that the crystallization setup contains sufficient amount of protein. Equation (2) provides an estimation of the minimal volume of protein V_{min} solution that should be used:

$$V_{min} \geq \frac{V_{crystal} \times N_{asymmetric\ unit} \times N_{protein\ chain} \times MW}{V_{unit\ cell} \times N_A \times C} \quad (2)$$

where $V_{crystal}$ is the target volume of the fully grown crystal, $N_{asymmetric\ unit}$ is the number of asymmetric units for the expected space group, $N_{protein\ chain}$ is the number of protein chains in the asymmetric unit, MW is the molecular weight of the protein, $V_{unit\ cell}$ is the volume of the unit cell, N_A is the Avogadro number and C is the protein concentration (in mg mL⁻¹). (A protein solution volume calculator using this equation is provided as Supplementary Material.) In using this equation to plan crystallization experiments, one assumes that only one crystal will grow per drop and that all of the protein in solution crystallizes. The estimation also relies on accurate protein concentration determination.

The readers are referred to the recent review by McPherson and Gavira [81] and to Gavira's review (*this issue*) for detailed protein crystallization principles. The most commonly utilized crystallization method for neutron crystallography is vapor diffusion, with 35 out of the

85 neutron protein structures deposited in the PDB solved from crystals grown by vapor diffusion. Large 10-25 μL hanging drops were used only for aldose reductase and diisopropyl fluorophosphatase [13, 40, 41, 56, 57]. Sitting drop crystallization allows for larger volume drops to be setup easily compared to hanging drops. Sitting drop volumes reported range from 20 μL to 100s μL . Sandwich box setups (Hampton Research) consisting of a siliconized nine-well glass plate placed inside a sealed plastic box, are typically used to set up larger ($> 50 \mu\text{L}$) volume sitting drops. According to the manufacturer, each well is capable of holding drop sizes up to 800 μL , and the box, which serves as precipitant reservoir, can hold up to 25 mL of crystallization solution. The largest reported sitting drops were setup with a total volume of 400 μL [17, 82]. Neutron structures solved from crystals grown using the sitting drop vapor diffusion method include carbonic anhydrase II, DHFR, and transthyretin [38, 82, 83]. Despite the fairly high success rate of the sitting drop vapor diffusion techniques, it should be noted that in about two thirds of the cases, the sitting drops are micro- or macroseeded as described below (also see Table S1).

Batch crystallization is a useful alternative method with about one third of the deposited structures reporting this technique (Table S1). Batch crystallization volumes for neutron crystallography span from 10s of mL in the early days of protein crystallization [84-86] to volumes more compatible with modern protein production of 100s of μL [20] and even down to 10 μL in a combined batch/seeding crystallization approach such as that used by Matsumura *et al.* [87] to crystallize the HIV Pr-KNI-272 complex. The batch method is usually applied to proteins available in large quantities such as xylose isomerase [33, 46]. However, large batch crystallizations are not a requirement during optimization for this approach to be successful [88, 89]. In fact, the batch method presents the distinct advantage over other crystallization techniques that scaling to larger volumes does not affect the kinetic and thermodynamic factors that regulate the crystallization process. Crystallization conditions optimized using small batch volumes or even by high-throughput micro-batch screening can readily be scaled up.

Dialysis and counter diffusion have been used to a lesser extent for neutron protein crystallography. Dialysis with protein solution volumes of 200 μL and above has been performed for concanavalin A using dialysis tubing [90-92], insulin using a micro-dialysis chamber [93] and P450cam using dialysis buttons (Hampton Research) [31]. Smaller volumes (50-100 μL) were dialyzed using dialysis buttons for cytochrome c peroxidase [94, 95] and insulin [96, 97]. A customized counter diffusion apparatus was successfully used to grow large inorganic pyrophosphatase crystals suitable for neutron data collection [98, 99]. Dialysis, batch, and counter diffusion setups can be supplemented with micro- and macro-seeding and “crystal feeding”. In crystal feeding fresh protein solution is added to a crystallization drop containing a crystal that appears to have stopped growing. The crystal is typically “fed” several times. This method was applied to *Achromobacter* protease I [100]. Special attention should be given to the crystal habit response to the feeding process as the etching of the crystal surface that may occur can lead to further nucleation or growth of a multi-lattice crystal.

Seeding techniques are powerful as they offer the opportunity to decouple nucleation from growth. Decoupling these two steps can be instrumental in growing large single crystals. Just under a third of the crystals used to collect neutron data set deposited in the PDB used micro- or macroseeding illustrating the usefulness of this approach (Table S1). Seeding is used for both X-ray and neutron crystallography when initial drops do not produce crystals with sufficient volume or diffraction quality. For example, micro-seeding was necessary to grow a large single crystal of aldose reductase [13]. Macroseeding was successfully applied to the thrombin-bivalirudin complex [101], cholesterol oxidase [48], HIV Pr [16], rubredoxin [102], elastase [103] and the recently determined structure of farnesyl pyrophosphate synthase [104, 105] (Table S1). In the last case, spontaneous nucleation and crystal growth was conducted in large 400 μL sitting drops that produced crystals with volumes up to 0.1 mm^3 . These crystals were then used to macroseed 60 μL drops in which one crystal reached a final volume of 3.5 mm^3 . Interestingly, Bennett *et al.* [106] grew large crystals of DHFR–methotrexate by vapor diffusion using large sitting drop while Wan *et al.* [22] combined both micro- and macroseeding with vapor diffusion to grow DHFR–folate–NADP(+) crystals suitable for neutron diffraction.

While protein concentration, precipitant type and concentration, and pH are the most varied crystallization parameters during optimization, temperature can also be an efficient parameter to vary [89]. By changing the crystal growth temperature, one can drive the protein solution from a nucleating state to a metastable state or maintain the solution in the metastable state to promote continued crystal growth. This can be performed using sophisticated equipment such as the temperature-controlled batch crystallization device developed and applied to urate oxidase by Budayova-Spano *et al.* [51, 107]. On the other hand, simply transferring crystals trays between incubators with different temperature set points or changing the incubator temperature by steps or continuous gradients provides straightforward options to vary temperature, though in a less controlled fashion [16, 87].

As discussed above, titratable hydrogen atoms present in the crystal should be exchanged to deuterium prior to data collection. Soaking, vapor diffusion, or crystallization in solution prepared with D_2O can be used for H/D exchange. Crystallization in D_2O solutions is a straightforward way to achieve H/D exchange, but this approach often does not produce crystals suitable for neutron diffraction or requires re-optimization of conditions. Soaking is the most efficient method for H/D exchange after crystal growth. However, crystals grown in H_2O solution may suffer damage (*e.g.*: cracks) from soaking in a fully deuterated solution. Gradual increases in D_2O concentration of the soak solution may help prevent such damage. A stepwise increase in D_2O concentration was successfully applied to DHFR crystals [106]. A gentler approach to H/D exchange is vapor diffusion. Vapor diffusion H/D exchange can be performed by replacing the hydrogenated mother liquor in the reservoir with an artificial deuterated mother liquor [108]. The well-to-drop ratio must be at least 10, and the D_2O solution should be replaced 3 times or more to maximize the exchange. If room temperature data collection is planned, H/D vapor exchange can occur inside the capillary in which the crystal is mounted as shown in Figure

4. The largest possible volume of deuterated solution “plug” must be used for exchange in capillaries, and this plug must also be repeatedly changed for new solution. For both the soak and vapor diffusion, longer exchange times permit exchange in buried area of the protein further and result in greater deuterium incorporation.

There is no systematic use of one exchange approached over another; the method used depends largely on the experimenter. However, data collection may restrict the choices. If data collection at cryogenic temperature is planned, soaking in or vapor diffusion with a deuterated reservoir will be the safest approaches. For room temperature data collection, the crystals should be mounted and exchanged in a quartz capillary since borosilicate glass absorbs neutrons (Figure 4). Uses of thin and thick wall capillaries are both reported in the literature. While thick wall capillaries generate higher background, they are sturdier. The choice of the type of capillary is again an experimenter’s preference.

Instrumentation, Data Collection, and Processing

Neutron crystallography is an inherently flux-limited technique. Available neutron fluxes are several orders of magnitude less than the X-ray flux available from modern in-house generators and synchrotrons. This limitation is the primary impetus for increasing the scattering power of the protein crystal through the isotopic labeling and crystal growth techniques discussed in the previous sections. Low available flux also results in the long exposure times required to measure a neutron diffraction dataset to sufficient resolution and completeness. While complete X-ray datasets can be measured in seconds or less at high-intensity X-ray sources, neutron datasets typically require days to weeks for collection. However, through recent advances in instrumentation and labeling it has become possible to obtain useful neutron datasets in a single day or less in special cases.[36]

Table 2 lists current single crystal diffractometers designed for neutron protein crystallography at neutron scattering facilities worldwide. In addition to these instruments, other multi-purpose single crystal diffractometers, such as the D19 instrument at ILL, have also been used for protein crystallography [5, 28, 37, 38]. Instruments can be divided into three groups based upon mode of operation, namely monochromatic, quasi-Laue (also known as “pink-Laue”) and time-of-flight (TOF) Laue modes. All instrument detector arrays are designed to maximize coverage of reciprocal space so that a large number of stimulated reflections that can be recorded simultaneously whether the instrument uses mono- or polychromatic incident radiation. Typically, neutron protein crystallography beam lines take advantage of longer wavelength neutrons to maximize the intensities of the diffraction peaks which are proportional to the square on the incident wavelength, as shown in Equation (1), and to reduce spatial overlap according to Bragg’s law ($2d * \sin(\theta) = \lambda$). At the wavelength typically used for X-ray crystallography ($\lambda \leq 1.54 \text{ \AA}$), the diffracted beams lie on cones in the forward scattering direction, and two-

dimensional detectors are installed downstream of the crystal. At the longer wavelengths ($2.3 \text{ \AA} < \lambda < 6.0 \text{ \AA}$) used by neutron diffractometers, the high resolution diffraction also occurs in the backscattering direction. Instrument detector arrays approximate cylindrical or spherical geometries to record both forward- and backscattering reflections.

Existing monochromatic and quasi-Laue instruments are installed at reactor neutron sources and take advantage of the high time-integrated neutron flux provided by these facilities. Monochromatic instruments operate analogously to standard X-ray macromolecular diffractometers with the exception of longer incident wavelengths. Quasi-Laue instruments accept a defined, broad range of incident neutron wavelengths ($\Delta\lambda/\lambda_{\text{mid}} \approx 25\text{-}60\%$) that greatly increases the number of stimulated reflections at each crystal setting relative to monochromatic mode. The broad wavelength bandpass therefore is a more effective use of neutrons available from the reactor source, and the configuration also avoids the need for a monochromator crystal upstream of the sample that would inevitably have less than 100% reflection efficiency at any selected wavelength. Quasi-Laue represents a compromise on the amount of observed incoherent background with it being more than that observed with monochromatic radiation but less than that observed with truly “white-beam” radiation. While the monochromatic geometry produces data sets of higher quality compared to the quasi-Laue geometry (either for X-rays or neutrons) for neutron protein crystallography the quasi-Laue configuration has the unique advantage of allowing smaller crystals and/or shorter exposure times to be used [109].

Unlike reactor sources, accelerator-driven spallation sources provide neutrons in discrete pulses, and this time structure permits the use of a TOF Laue mode for macromolecular diffractometers. A relatively large wavelength range can be accepted by the instrument to maximize the number of stimulated reflections. The detectors record the positions, intensities and time of incidence for each scattered neutron from each pulse. The neutron time of flight can then be converted to the corresponding wavelength, and the observed quasi-Laue diffraction pattern can be “binned” or separated into reflections and background for each incident wavelength. TOF methods, therefore, provide both the increased number of reflections of quasi-Laue mode and the reduced background of monochromatic mode.

Table 2. Diffractometers for neutron protein crystallography.

Instrument	Source	Mode of Operation	Wavelength (Å)	Status	Refs.
BIX-3	JRR-3M, Japan	monochromatic	2.35	operating	[110]
BIX-4	JRR-3M, Japan	monochromatic	2.6	operating	[111]
Bio-C	HANARO, South Korea	monochromatic	1.95	commissioning	[112]
BIODIFF	FRM II, Germany	monochromatic	2.4	operating	[113]

LADI III	ILL, France	quasi-Laue	3.0 – 4.0 3.5 – 4.7 4.0 – 5.4 4.5 – 6.1	operating	[39]
IMAGINE	HFIR, USA	quasi-Laue	2 - 3.0 2.8 – 4.0 2.8 – 4.5 3.3 – 4.5	operating	[114]
PCS	LANSCE, USA	TOF-Laue	0.7-7.0	decommissioned	[115]
MaNDi	SNS, USA	TOF-Laue	2.16-4.3	operating	[116]
iBIX	J-PARC, Japan	TOF-Laue	0.7-3.85	operating	[117]
NMX	ESS, Sweden	TOF-Laue		design	[118]

Data collection at two or more κ angles is essential when using longer wavelength radiation because the curvature of the Ewald sphere is such that the blind region is significant at any one κ setting. In addition, instruments using a cylindrical detector geometry (BIODIFF, BIO-C, BIX-3, BIX-4, IMAGINE, and LADI-III) have large, systematic vacancies in their coverage of reciprocal space along the cylindrical long axis. Reflections absent due to detector coverage can be recovered by using more than one κ angle.

Sample handling for neutron diffraction experiments is generally different from that for both in-house and synchrotron X-ray experiments due to the non-destructive nature of the neutron radiation. Neutrons having wavelengths of $< 10 \text{ \AA}$ ($< 50 \text{ meV}$) do not induce ionization in biological samples. Therefore, cryogenic sample temperatures are not necessary to protect against radiation damage in the protein crystal, and the majority of experiments are performed at ambient temperature. While room temperature data collection is typical, cryo-crystallography does, however, reduce thermal disorder in protein crystals and makes possible the study of reaction intermediates and temperature sensitive systems [119]. As reviewed by Coates *et al.* [120] nitrogen cryostreams have been installed on a subset of neutron diffractometers to perform measurements at 100 K with sample mounting equipment standard for X-ray cryo-crystallography. For either temperature condition, crystal mounting and alignment are often performed manually; however, the newest instruments such as MaNDi do incorporate motorized sample handling and positioning under user control [116]. Given that data collection time exceeds days, manual centering is not a significant overhead. Upon completion of data collection,

particularly at ambient temperature, the crystal is recovered. This same crystal can then be used for additional neutron experiments or to collect an X-ray dataset for joint refinement of X-ray and neutron data (see *Structure Refinement*).

Diffraction data from reactor-based instruments can be indexed and integrated using software packages developed for X-ray crystallography with appropriate modifications to account for the instrument's detector geometry. Software packages used include Lauegen [121], d*TREK [122, 123] and DENZO [124]. Handling the additional TOF information recorded by spallation-source instruments requires the development of dedicated data reduction packages [125, 126]. Unlike monochromatic data, polychromatic datasets (either for X-rays or neutrons) must be wavelength-normalized to account for the spectral shape of the incident beam. Software used to wavelength normalize data are also adapted from X-ray Laue crystallography software packages [121, 127]. Data scaling is then performed with routine protein crystallography software such as SCALEPACK [124] or SCALA [128].

Structure Refinement

Structure refinement is carried out against neutron data alone or simultaneously against both X-ray and neutron data. The latter protocol is referred to as joint refinement or X/N refinement [129]. The choice of one method of refinement over the other may be influenced by the neutron dataset resolution and completeness but is ultimately the experimenter's choice. The number of parameters to be refined for a neutron structure is considerably greater than for an X-ray structure: the number of atoms is nearly doubled, and ~25% of the hydrogen positions require H/D-occupancy refinement. However, typical neutron datasets contain fewer unique reflections than their X-ray counterparts because of lower completeness and resolution. Refining a model against both X-ray and neutron data significantly increases the data-to-parameter ratio and reduces the risk of “over-fitting” the available data during refinement [130]. Joint refinement is widely used for neutron structure refinement against data collected from partially deuterated proteins at resolutions below 2.2 Å. At these moderate resolutions, cancellations of neutron density for CH, CH₂ and CH₃ groups may obscure the direct modeling of side chains [61, 130]. In joint refinement the electron density maps are used to refine the positions of heavy atoms while the corresponding neutron density maps are only used to refine hydrogen atom positions [129, 130]. Examples of proteins for which both refinement against neutron data alone and joint refinement have been applied include photoactive yellow protein (PYP), transthyretin, and DHFR. Yamagushi *et al.* [131] refined the neutron structure of PYP at 1.5 Å against neutron data alone while Fisher *et al.* [132] performed joint refinement to determine the PYP neutron structure at 2.5 Å. Both refinement strategies have been applied to neutron structures of transthyretin at equal resolution (2.0 Å) [38, 133] and to the neutron structures of DHFR at 2.0 Å [22] and 2.17 Å [21] resolutions. Protein perdeuteration prevents density cancellation by hydrogen atoms, but joint refinement is still broadly applied to data collected from perdeuterated

crystals. The neutron structures of perdeuterated aldose reductase [41], transthyretin [38], and type III antifreeze protein [67] were jointly refined. In contrast, structures of beta lactamase [19, 20], myoglobin [65] and rubredoxin [36] were successfully solved using neutron data alone.

Joint refinement requires that the X-ray data set be collected at the same temperature as the neutron data set. Collecting both data sets at the same temperature is essential as temperature alters aspects of the protein structure or of the crystal itself [134-138]. X-ray-induced radiation damage can be more severe in crystals measured at room temperature and can produce significant structural artifacts. Radiation damage can be minimized at room temperature by using short exposure times and strong attenuation of high-intensity incident beams. While the X-ray and neutron data set would ideally be collected from the same crystal for joint refinement, the X-ray dataset is most often measured from a smaller crystal grown in the same drop or under the same crystallization conditions as the neutron crystal. Large crystals typically produce lower quality X-ray data due to absorption, and radiation damage from X-ray exposure would make the large crystal unsuitable for subsequent neutron parametric studies (Knihtila, Mattos, and Meilleur, unpublished results).

Currently, there are three refinement programs that can refine neutron protein diffraction data: CNSsolve [62, 139], phenix.refine [61, 130, 140], and SHELXL [141, 142]. The phenix.refine module of the PHENIX software package [140] is capable of refining X-ray, neutron, and joint X-ray/neutron data [61]. The familiarity of phenix.refine to the crystallography community has made it the most used package for neutron structure refinement. Crystallography and NMR system (CNS) was originally developed as an online server to aid structure determination from solution NMR and X-ray crystallography data [139, 143]. A neutron diffraction patch, termed nCNS, can be added to CNS to perform refinement of neutron or joint X-ray/neutron data with CNSsolve [62, 130]. nCNS has been used for 19 deposited neutron structures. SHELXL has been used to refine neutron structures of crambin [144], xylose isomerase [27, 30], DHFR [21] and endothiapepsin[145]. Recently Gruene *et al.* [142] modified SHELXL to include specific instructions for neutron macromolecule structure refinement.

Refinement against neutron data closely follows X-ray refinement protocols, and neutron density maps can be visualized using the program COOT [146, 147]. The starting model is a structure determined at the same temperature using X-ray data alone that has been stripped of all ligands and water molecules. Deuterium or hydrogen atoms are added at all non-titratable positions for perdeuterated or hydrogenated proteins, respectively. Coordinate file editing can be performed manually or automatically by utilities such as phenix.ready_set [148]. Deuterium and hydrogen atoms, each with half occupancy, are usually added to the titratable positions when the crystal is H/D exchanged after crystallization. Deuterium atoms with full occupancy can be added if the protein was crystallized from deuterated buffer [65, 67, 86, 113, 114, 145, 149]. In this latter approach incompletely exchanged sites will show a negative density peak in the neutron $F_o - F_c$ map indicating that the site is partially occupied by hydrogen [36]. At this initial

state of refinement, titratable groups are assumed to be in their expected protonation state based on the “normal” pK_a value of the individual amino acid in solution.

An initial rigid body refinement and successive coordinate, atomic displacement parameter and occupancy refinements are performed in alternation with local rebuilding of the model including the addition of small molecules. Water molecules can be modeled as O, OD, OD⁻, DOD, and DO₃⁺ depending on their degree of rotational anisotropy, neighboring groups, and ionic state [32, 37, 150]. Water molecules can be oriented automatically by the refinement software. However, visual inspection of the model and neutron density maps should be performed to confirm the automatically refined orientations. Experimenters should consider manual orientation of the water molecules to form hydrogen bonds with accepted geometries when possible[151]. Map observations will also inform the modeling of titratable group protonation states. Careful interpretation of the maps is essential to model “abnormal” or “perturbed” ionization states. Here the neutron $F_O - F_C$ maps are again used to model hydrogen and deuterium atoms. Positive map features directly indicate protonation of carboxylate, hydroxyl, amine and imine groups. Proper orientation of these groups, guided by the neutron $F_O - F_C$ maps, often clearly defines hydrogen-bond networks that may be ambiguous if only the electron density maps are considered. A negative $F_O - F_C$ feature at a titratable site indicates an overestimation of deuterium occupancy at this site. Deuterium atoms are readily visible as positive features in neutron $2F_O - F_C$ maps at resolution ≤ 2.5 Å. It should be noted that while hydrogen atoms included in the coordinate file will contribute to negative features in the neutron $2F_O - F_C$ map, hydrogen atoms are usually only visible at higher resolutions (≤ 2.0 Å).

Perspective

Atomic comprehension of protein chemistry requires knowing the simultaneous locations of numerous hydrogen atoms. Specifically for enzymes, the interactions driving chemistry generally cannot be unambiguously deduced solely from knowledge of the protonation states of individual titratable side chains within the active site [4]. It is widely accepted that remote amino acids [152-156] or water molecules [157-159], whether buried in the protein structure or bridging the active site to the hydration shell, play essential roles in protein chemistry. At the active sites of enzymes, both the chemical identities of ligands, substrates and water molecules (H_2O , OH^- , or H_3O^+) and the hydrogen bond networks formed must be known for a complete mechanistic understanding. Furthermore, the modulation of active site pK_a 's by the local electrostatic environment must be understood.

The neutron crystal structures of human farnesyl pyrophosphate synthase (FPPS) in complex with the clinical inhibitor risedronate and of human transthyretin (TTR) illustrate these points. FPPS catalyzes condensation of dimethylallyl pyrophosphate (DMAPP) with isopentyl pyrophosphate (IPP) yielding geranyl pyrophosphate (GPP) which further condenses with a second IPP molecule yielding farnesyl pyrophosphate. Nitrogen-containing bisphosphonates (N-BP), such as risedronate, inhibit FPPS by occupying the DMAPP/GPP binding site [160]. N-BPs bind the FPPS active site with a high affinity that was proposed to result from a protonated nitrogen mimicking a carbocation intermediate of the FPPS condensation reaction [161, 162]. The X-ray crystal structure of the FPPS–risedronate complex demonstrated that the risedronate pyridine nitrogen was positioned within hydrogen bonding distance of the Lys200 backbone carbonyl and the Thr201 hydroxyl group. The neutron crystal structure of H/D-exchanged FPPS–risedronate at $\text{pD}=5.0$ determined by Yokoyama *et al.* [104] confirms the existence of these hydrogen-bond interactions. A deuterium atom binds to the pyridine nitrogen and participates in a bifurcated hydrogen bond with Lys200 and Thr201. This protonation agrees with prior studies of pK_a 's for the risedronate titratable groups in solution. However, the neutron structure revealed that both risedronate phosphonate groups are fully deprotonated when the drug is bound to FPPS despite the first deprotonation of the phosphonates in free risedronate having a $\text{pK}_a > 10.5$ [163, 164]. The phosphonate groups together coordinate three Mg^{2+} ions and participate in hydrogen bonds with Arg112, Lys200 and Lys257 sidechains and four ordered water molecules. While unexpected, phosphonate deprotonation agrees well with a FPPS mechanism in which a basic pyrophosphate oxygen, stabilized by Arg112 and Lys200, abstracts the IPP 2R proton promoting nucleophilic attack of the carbocation intermediate [161, 162]. These two mechanistic insights resulted directly from unambiguous delineation of the residue, ligand, and water protonation states at the enzyme active site by the neutron crystallography experiment.

The neutron structures of human transthyretin (TTR) reveal key hydrogen bond networks and pH sensitivity leading to a new understanding of substrate binding and amyloid formation. TTR is present in the blood, where it binds retinol and thyroxine (T_4), and in the cerebrospinal

fluid, where it binds T₄. The TTR protein is a homotetramer, or a dimer of dimers, held together through an extensive network of hydrogen bonds and hydrophobic interactions. Partial denaturation of TTR, which occurs in acidic conditions, leads to aggregate structures that include amyloid fibril formation. Previous TTR X-ray crystal structures did not display significant conformational changes when the pH was altered [165, 166]. The neutron structure solved by Yokoyama *et al.* revealed an extensive hydrogen bond network connecting six residues and four water molecules located along both the monomer-monomer and dimer-dimer interfaces [133]. One of the residues involved in the hydrogen bond network is the singly protonated His88, which stabilizes the dimer-dimer interface. When the protonation state of His88 is altered, the interfacial hydrogen bond network is destabilized. Comparison of the neutron structure solved at pD 7.4 with a previously solved X-ray structure at pH 4.0 shows that the salt bridge between Lys76 and Glu89, the hydrogen bond between Asp74 and Ser77, and the hydrogen bond network including His88 are compromised at low pH. Haupt *et al.* also described the extensive hydrogen bond network between monomer-monomer interface in their structures leading to the conclusion that the dimer of TTR is the building block to amyloid fibril formation [38]. The neutron structures of TTR increased the understanding of pH sensitivity and suggested a potential mechanism of amyloid formation.

In the last year two neutron structures revealed unexpected protonation states of key states of established enzymatic mechanisms. The neutron structure of the small GTPase RAS further illustrates the uniquely rich information content provided by a single neutron diffraction experiment [108]. This study suggests that the γ -phosphate group of the GTP substrate remains protonated at physiological pH when bound at the active site of RAS in the ground state. Efforts to elucidate the GTP hydrolysis mechanism of RAS have relied on the assumption that the γ -phosphate group of GTP bound at the active site of the enzyme would be deprotonated as is observed in solution. The neutron structure revealed for the first time the modulation of the pK_a of γ -phosphate group by the RAS active site microenvironment and sets a new stage for future investigation. Neutron structures are usually determined at room temperature but neutron data collection at cryogenic is necessary to characterize transient states. Intermediate freeze trapping was successfully applied to cytochrome c peroxidase to determine the structure of ferryl heme intermediate and unambiguously determined the protonation state of the iron-oxo species (also known as compound I) [94]. The study demonstrates that compound I is deprotonated supporting earlier data. Comparison of the room temperature neutron ferric cytochrome c peroxidase structure and of the cryo neutron structure of compound I critically revealed that the heme distal histidine, become protonated. Hence both the neutron studies of Ras and cytochrome c peroxidase state a new stage for future calculations and modelling.

Readers are referred to the recent review by Golden and Vrielink [167] for additional examples of neutron crystallographic structures providing insights into hydronium ions, oxyanion holes, low barrier hydrogen bonds, and mapping protein–ligand interactions.

NMR spectroscopy, ultra-high resolution X-ray crystallography and neutron crystallography contribute to a global understanding of protonation. Taken individually, each technique has specific limitations. Acid–base titrations can be monitored by NMR spectroscopy to determine pK_a values directly [168-170]. However, pH titration methods can only be applied to proteins that are stable over a wide range of pH, and the experiment does not simultaneously report on the residue conformation. Residues surrounding titratable sites also have chemical and structural behaviors sensitive to pH that may complicate chemical shift assignments and pK_a determination. In addition to probing pK_a 's, long residence-time water molecules can be localized using NMR spectroscopy, but water molecule orientations are difficult to determine [171]. For X-ray crystallography, the weak X-ray scattering power of hydrogen atoms inherently limits the available experimental information. The protonation state of some titratable sites can be determined indirectly from X-ray structures by covalent bond geometry analysis. High resolution X-ray crystallography proves not to be the most suitable technique to visualize hydrogens directly [40, 69]. Neutron protein crystallography directly identifies H/D atoms, but is inherently limited by the flux of the available neutron beams. However, when large crystals can be grown, a single neutron structure informs simultaneously on all aspects of catalytic activity: structural conformation and protonation state at the active site, conformation and protonation of remote residues, chemical identities and orientations of water molecules, and the organization of hydrogen-bond networks.

Neutron crystallography is a powerful complementary approach to NMR spectroscopy and X-ray crystallography. Neutron crystallography and NMR spectroscopic measurements following the titration of tyrosine residues were elegantly combined in a study of carbonic anhydrase by Michalczyk *et al.* [26]. NMR revealed the perturbed pK_a of Tyr 7, and the neutron crystal structures provided a structural context. Similarly, sub-atomic resolution X-ray and neutron crystallography were combined to describe in further detail the catalytic mechanism of DHFR [22].

As we have discussed in this review, neutron protein crystallography does present a number of technical challenges. Deuterium incorporation is essential for neutron diffraction experiments, but, the studies reviewed here illustrate that the incorporation required is readily achievable. The exchange of titratable hydrogen atoms suffices when full perdeuteration is not practical. Many crystals will likely endure vapor diffusion H/D exchange. Data collection and structure refinement methods are now well established, and various strategies can be selected to best suit the crystal system and scientific question being addressed.

Growing large protein crystals remains as the final hurdle for the widespread pursuit of neutron protein crystallography. Clearly, all proteins are not amenable to the growth of large crystals. However, a significant fraction of proteins that produce crystals with X-ray diffraction limit $\leq 1.5\text{\AA}$ (currently, approximately 5000 proteins meet this criteria), or that have been investigated extensively as reflected in the PDB, is likely to be amenable to neutron diffraction. The foreseen brightness of neutron beams at facilities currently being built (European Spallation

Source) or proposed (Second Target Station at the Spallation Neutron Source), combined with novel instrument concepts will contribute to further lowering the crystal size requirement. The application of polarization techniques for neutron protein crystallography will open up new opportunities in studying fully hydrogenated protein crystals [172, 173]. These developments will undoubtedly expand the opportunities to explore protein chemistry at the atomic level.

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