Chapter VI

Progress toward Determining the Location of Hydrogen Atoms in the MoFe Protein using Neutron Diffraction

VI.A. Abstract

The heart of nitrogenase activity involves the transfer of electrons and hydrogen atoms between and around the metal clusters. Although X-ray crystallography is a powerful technique with high throughput, ultra-high resolution structures (<1.0 Å) are required to observe hydrogen atoms, which have thus far not been attained for nitrogenase. Alternatively, neutron diffraction may be used to observe hydrogen and deuterium atoms in a crystal structure at ~2 Å resolution. This work establishes conditions and crystal size required to obtain adequate neutron data sets of partially deuterated MoFe protein to observe the location of hydrogen and deuterium atoms. Using a spallation neutron source and a high flux isotope reactor at Oak Ridge National Laboratory, initial room temperature screens on partially deuterated Cp1 crystals show diffraction resolution >4 Å; cryogenic screens exhibit poorer resolution and diffraction quality. The next step is to increase the crystal size to ~1 mm^3, which should be sufficient to obtain ~2 Å resolution data and determine the location of hydrogen and deuterium atoms in the protein. This information would provide insight into where hydrogen is stored and how it is accessed during turnover in the MoFe protein. The long term goal of this project is to obtain neutron diffraction data of non-resting state structures of the MoFe protein and compare the location of hydrogen atoms to that of the resting state structure in order to observe how hydrogen moves around the cofactors during substrate turnover.

VI.B. Introduction

Nitrogenase catalyzes the reduction of atmospheric dinitrogen into its bioavailable form, ammonia, in a process called nitrogen fixation. The enzyme consists of the Fe and MoFe proteins. The MoFe protein contains two types of metal clusters, the [8Fe:7S] P-cluster and the [7Fe:Mo:9S:C:*R*-homocitrate] FeMo-cofactor, the latter of which is the active site. Two of the most commonly studied bacterial species that express nitrogenase are *Azotobacter vinelandii* (Av) and *Clostridium pasteurianum* (Cp), for which the MoFe proteins are named Av1 and Cp1, respectively. Due to the complexity of nitrogenase, the mechanism of biological nitrogen fixation remains elusive. High resolution X-ray structures of the MoFe and Fe proteins have revealed the identity and location of all atoms heavier than hydrogen with structures refined to 1.0 and 1.08 Å for Av1 and Cp1, respectively.[22,64] The high resolution X-ray data, combined with electron nuclear double resonance spectroscopy, was used to identify the central atom in the FeMo-cofactor as a carbon species.[22,64] The electron density of this atom was obscured in all previous structures due to perturbations from the neighboring electron-dense Fe atoms. Additionally, the binding mode of the inhibitor, carbon monoxide, to the FeMo-cofactor of Av1 was determined using X-ray diffraction.[37] This was the first observation of any species bound to the nitrogenase active site.

Studies on the binding, activation, and cleavage of N_2 have concluded that the participation of protons and/or hydrides at different steps could be highly important to elucidating the complete mechanism of biological nitrogen fixation.[22,34] Neither 1.0-Å resolution X-ray data nor data from other techniques have established the positions of hydrogens on the metalloclusters or solvent that are critical for proton transfer processes in nitrogenase. X-ray diffraction does not usually detect hydrogen because it involves the detection of X-rays elastically scattered from electrons in a crystal to yield an electron density map; the scattering is

roughly proportional to the atomic number. Additionally, X-ray radiation damages protein crystals, which limits the dose of radiation one can apply to a crystal. Liquid state NMR can also be used to determine protein structure, although this becomes increasingly difficult with larger molecules due to overlapping signals; thus, atomic resolution of structures cannot be reasonably solved for proteins as large as the 220-kDa MoFe protein.[119]

Given current technology, the most suitable method to determine the location of hydrogen atoms is neutron diffraction combined with X-ray crystallography. Neutron diffraction data are gathered by detecting neutrons that are elastically scattered from nuclei in crystallized proteins. Fourier transform of the reflections from diffraction patterns gives a nuclear density map to which a protein structure can be modeled. Deuterium and hydrogen in a protein structure can be observed at resolutions around 2-3 Å, depending on the protein size and deuteration level.[120] Hydrogen and deuterium are easy to distinguish from each other because hydrogen has a negative incoherent (inelastic) scattering length of -3.74 10^{-15} m while deuterium has a positive coherent (elastic) scattering length of 6.67 10^{-15} m.[121] The incoherent scattering from hydrogen results in high background noise, but the signal-to-noise ratio can be reduced by soaking the crystals in D_2O-based solutions, crystallization in D_2O-based solutions, or growing the bacteria in D_2O-replaced media – techniques that offer increasingly better deuteration and therefore stronger coherent scattering signals.[122] Another advantage of neutron diffraction is that it does not cause radiation damage to the crystal.[123]

Despite the advantages of neutron diffraction, the technique is still in its infancy: the first neutron structure, that of myoglobin, was determined in 1969,[124] and the second structure, trypsin, was solved in 1980.[125] Currently, there are less than 100 neutron structures in the Protein Data Bank, compared to approximately 114,000 X-ray structures. The use of neutron diffraction is becoming increasingly popular due to ongoing technological progress allowing

smaller and smaller crystals with larger unit cell volumes to be imaged. Neutron data up to 1.1-Å resolution and with up to 95% visibility of hydrogen and deuterium have been reported.[126] Furthermore, newly engineered instruments with higher flux and increased detector sensitivity have decreased the data collection time period from months to days or weeks, depending on the size of the crystal, crystal symmetry, volume of the unit cell, and deuteration level.[127] Some instruments also provide neutron time-of-flight data so that the data can be binned for optimal signal-to-noise. When using neutron diffraction, large crystal sizes (>0.3 mm^3) and smaller unit cell volumes (<1 x 10^5 $Å^3$) are advantageous to compensate for the inherent weak flux of neutron sources, which is several orders of magnitude weaker than that of synchrotron X-ray sources.[123] The structure refinement of neutron datasets has also been an issue because there are roughly twice as many parameters compared to X-ray data, but new refinement programs specifically for solving neutron structures are available.[123]

The goal of this project is to observe the position of protons and/or hydrides near the metal centers in the resting state Cp1 protein using neutron diffraction. This knowledge will (1) provide information on the overall dynamics of the enzyme by showing the exchangeability of protons on amino acid residues possibly involved in proton transport, and (2) dictate the required crystal size and deuteration level for adequate neutron diffraction. Looking ahead, this will inform neutron diffraction experiments on non-resting state Cp1, which will aid in the understanding of hydride intermediates occurring on the metal clusters.

VI.C. Instrumentation

The neutron diffractometers used in this work are IMAGINE and MaNDi, which are instruments on the High Flux Isotope Reactor (HFIR) and Spallation Neutron Source (SNS), respectively, at Oak Ridge National Laboratory (ORNL) in Oak Ridge, Tennessee. IMAGINE

provides a constant flux of neutrons from a Uranium-235 core with a flux of 2.5 x 10^{15} neutrons/cm^2/s. The instrument operates under quasi-Laue conditions, detecting neutrons with wavelengths between 2-10 Å. It is advantageous to detect longer wavelength neutrons because they scatter better than shorter wavelength neutrons. The detector is a neutron image plate (Gd_2O_3 doped BaF(Br.I):Eu^{2+}). MaNDi operates with a spallation source with a frequency of 60 Hz (~10^7 neutrons/cm^2/s). MaNDi is also quasi-Laue, but has a smaller range of neutron detection: 2-4 Å. The detectors on MaNDi are anger lithium glass scintillators. When a neutron collides with the detector, it causes emission of ~50 photons, which produces a current that is measured by the detector. Cumulative reflections can be observed in real time. Additionally, because time-of-flight data is stored for each neutron, data is binned according to neutron wavelength. Neutrons of higher wavelength are better scatterers and allow better spacing of low resolution reflections, but also lead to more noise. Thus, by binning the data, the signal-to-noise ratio can be optimized.

VI.D. Progress to date

VI.D.i. Growing crystals

Typical crystallization experiments included 11.5-15% PEG with molecular weight 3350 g/mol, 0.2-0.5 M magnesium chloride, 0.08 M Tris/HCl at pH 8.0, and 5 mM sodium dithionite in D_2O using the sitting drop, vapor diffusion crystallization method in an anaerobic chamber at room temperature. Crystals shaped as rectangular prisms started growing within 24 hours. Most reached maximum size within a couple weeks. No crystal degradation was observed for crystals left in the crystallization drop up to six months. Crystal growth required that the crystallization drop consist of a protein stock to reservoir solution ratio of 1:1 (typically 2.0 or 2.5 µL of each). It was found that more highly concentrated protein stock produced larger crystals.

Crystals sent to ORNL for screening were typically around 0.1 mm^3. Several seeding techniques were employed to increase crystal size, including streak seeding from crushed crystals, feeding (adding additional protein stock to wells containing a single crystal), and macro-seeding (soaking a single crystal in fresh reservoir solution to dissolve the outer layer of protein molecules and then transporting the crystal to a fresh protein drop). Only streak seeding was successful. Feeding sometimes increased crystal size but mosaicity decreased. Macro-seeding tended to partially dissolve the crystal.

VI.D.ii. Deuteration

Increased deuteration of a protein crystal leads to decreased noise from inelastic scattering off of hydrogen atoms. Partial deuteration was achieved by crystallizing the protein stock in deuterated solvents. With a 2 µL protein drop exposed to 1 mL of deuterated reservoir solution, the ratio of H_2O to D_2O is approximately 2:1000 – enough to replace all exchangeable protons with deuterium in the growing protein crystal. To achieve complete deuteration (perdeuteration), the bacteria must be grown in deuterated media with a deuterated carbon source. This is an expensive endeavor as well as challenging, since the use of D_2O instead of H_2O significantly impacts the kinetics of cell growth. Due to these limitations, only a few species of bacteria have been optimized for growth in deuterated media, none of which have been shown to express nitrogenase.

VI.D.iii. Mounting, storing, and shipping crystals in capillaries

A detailed description for mounting and storing crystals in capillaries is provided in Chapter II. Capillaries arrived intact and undamaged to ORNL when shipped via FedEx Ground and FedEx Air.

VI.D.iv. Determining crystal stability and ideal screening temperature

At the time of screening, both IMAGINE and MaNDi operated on room temperature conditions but only MaNDi could operate at cryogenic temperatures. Cryogenic temperatures are favorable because they protect nitrogenase crystals from oxidation and degradation. Since neutrons cause no radiation damage, room temperature neutron data collections are feasible given sufficient crystal stability. Crystals were assessed for diffraction resolution at 298 K and 100 K on an in-house X-ray generator (Table VI-1), which has fluxes comparable to neutron sources. The results show no significant difference in diffraction resolution. For comparison, crystals were also screened at the Stanford Synchrotron Radiation Lightsource (SSRL) at 100 K.

Collecting a full neutron data set on a MoFe protein unit cell with $P2_1$ symmetry would require a whole cycle of beam time (~23 days). Crystals must be stable for at least two months, to allow time for sample preparation, shipping, and data collection. Crystals stability in capillaries at room temperature was investigated. In this experiment, diffraction quality was assessed at various time points after transfer to capillary. It was found that crystals could not be exposed to the X-ray beam more than once, even if for a single screen shot, due to radiation damage. Therefore, 30 crystals from the same seeding wells were mounted in capillaries on the same day. Every few days, a fresh crystal was screened on the in-house generator. Crystals started exhibiting poor diffraction quality after about six weeks; thus, a room temperature neutron dataset would likely have to be made from two or more merged datasets.

Table VI-1. X-ray crystallographic data collection and refinement statistics

	Room temp, in-house	**100 K, in-house**	**100 K, SSRL**
Data Collection Statistics			
Resolution range (Å)	29.25 – 2.06 (2.06 – 2.17)	19.77-2.00 (2.03-2.00)	36.84 – 1.62 (1.66 – 1.62)
Wavelength (Å)	1.5418	1.5418	0.9537
Space group	$P2_1$	$P2_1$	$P2_1$
Unit cell constants	a = 69.97 Å b = 151.3 Å c = 117.8 Å α = 90.00° β = 103.5° γ = 90.00°	a = 69.4 Å b = 149.6 Å c = 116.7 Å α = 90.00° β = 103.8° γ = 90.00°	a = 69.4 Å b = 148.8 Å c = 116.4 Å α = 90.00° β = 103.9° γ = 90.00°
Unique reflections	138,737	149394 (7181)*	3311 (21193)*
Completeness (%)	94.7 (92.5)*	96.2 (93.4)*	99.3 (99.0)*
Redundancy	2.9 (2.8)*	7.7 (7.8)*	7.2 (5.1)*
$I/\sigma(I)$	6.2 (2.0)*	13.6 (3.0)*	39.1 (2.3)*
R_{merge}	0.128 (0.636)*	0.150 (0.696)*	0.030 (0.593)*
Refinement Statistics			
Protein residues	1950/1984	1950/1984	1950/1984
Mean B value (Å^2)	20.0	13.8	18.8
R_{work}	0.176	0.184	0.167
R_{free}	0.221	0.223	0.196
RMSD bond lengths (Å)	0.011	0.012	0.012
RMSD bond angles (°)	1.47	1.85	1.78
Ramachandran outliers	10 (0.52%)	5 (0.26%)	5 (0.26%)
Water molecules	494	1181	559

*Numbers in parentheses represent data in the highest resolution shell.

VI.D.v. Screening crystals

Screening was performed on IMAGINE using a beam size of 2.5 x 3.0 mm^2. A crystal with dimensions of 0.5 x 0.5 x 0.3 mm (0.075 mm^3) was subjected to neutron wavelengths of 2-10 Å, and the best diffraction was 7-8 Å resolution (Figure VI-1). Larger crystals (~0.1 mm^3) were screened using a restricted band pass, 2.8-4.0 Å, which yielded better diffraction quality and resolution (~4 Å); however, higher resolution and more complete diffraction patterns are still needed. Three months later, the crystals diffracting to ~4 Å were screened again on IMAGINE.

No diffraction was observed, and the crystals were noticeably lighter in color, indicating that oxidation had occurred. No obvious damage to the capillary was apparent. Any future data collections on IMAGINE would require multiple crystals to collect a complete dataset.

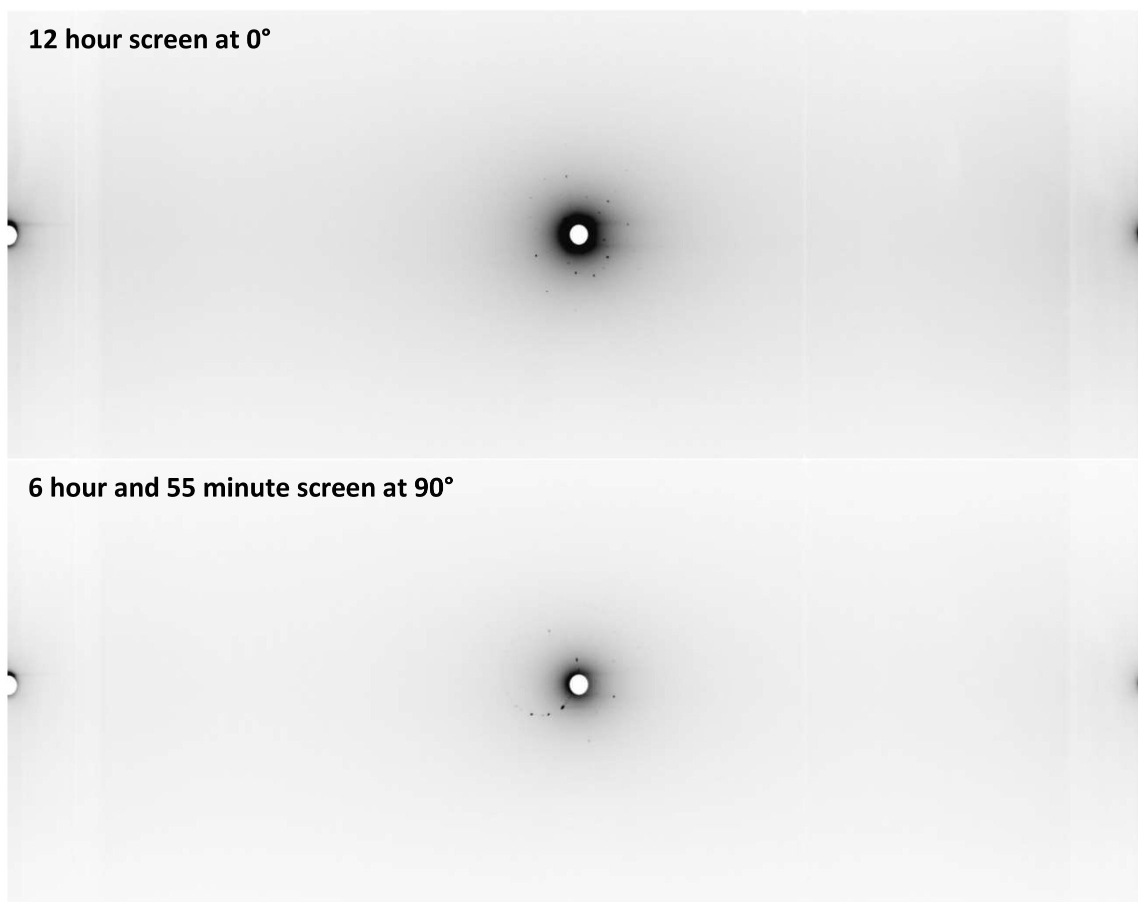

Figure VI-1. The best diffraction observed with Cp1 crystals on IMAGINE was 7-8 Å. Longer screen times yield better diffraction but increased noise. The screening images are cylindrical to reflect the shape of the detector.

Screening on MaNDi was performed to assess the potential of the cryogenic system. Despite our best efforts, we were unable to prevent ice buildup. After a 22-hour screen with a 1 mm beam aperture, 5-7 Å diffraction resolution was observed, but it was very noisy. Thus, room temperature neutron experiments are more favorable compared to cryogenic temperatures.

VI.E. Conclusions and next steps

Although Cp1 crystals are more stable at cryogenic temperatures, the cryogenic systems on MaNDi were not reliable at the time of screening. For room temperature data collection, stability tests indicate that multiple crystals would need to be imaged and their datasets merged to create a full neutron dataset. Regarding instrument selection, IMAGINE is preferred over MaNDi due to its ability to detect longer wavelength neutrons, which are better scatterers and therefore advantageous for proteins with large unit cells.

The immediate next step in this work is to increase crystal size to ~1 mm^3, which is a ten-fold increase from current crystal sizes. This scale increase necessitates a new approach to growing crystals, such as batch and large-protein drop (1 mL or more) vapor diffusion methods. These large scale crystallization methods significantly impact crystallization kinetics, so factors such as feeding, temperature, and protein-to-buffer ratios will have to be meticulously controlled during the scale-up process to determine conditions that facilitate large, single crystal growth. If the appropriate crystal size can be achieved and a whole neutron dataset resolved to at least 2 Å can be obtained, information on the location of hydrogen atoms on or near the cofactors may be observed. This will inform us on how hydrogen may be used and accessed during the course of substrate turnover, which would be invaluable information in the goal of elucidating the mechanism of biological nitrogen fixation.

VI.F. Acknowledgments

Many thanks to Dr. Flora Meilluer and Dr. Matthew Cuneo for their assistance in screening experiments on IMAGINE and MaNDi, respectively, and their input on this project.