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A history of neutrons in biology: the development of neutron protein crystallography at BNL and LANL

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The first neutron diffraction data were collected from crystals of myoglobin almost 42 years ago using a step-scan diffractometer with a single detector. Since then, major advances have been made in neutron sources, instrumentation and data collection and analysis, and in biochemistry. Fundamental discoveries about enzyme mechanisms, biological complex structures, protein hydration and H-atom positions have been and continue to be made using neutron diffraction. The promise of neutrons has not changed since the first crystal diffraction data were collected. Today, with the developments of beamlines at spallation neutron sources and the use of the Laue method for data collection, the field of neutrons in structural biology has renewed vitality.

1. Introduction

This very brief history of neutrons in biology will highlight just one aspect: the development of neutron protein crystallography from the beginning with the first look at hydrogen in myoglobin (Mb) through to current studies of enzymatic processes, drug binding, protein-hydration effects and dynamics. Such a brief history of neutron protein crystallography cannot possibly be inclusive and many important contributions have been omitted. However, there are a number of reviews and monographs that give more detailed information in selected areas (see, for example, Blakeley, 2009; Blakeley *et al.*, 2008; Niimura & Bau, 2008; Stuhrmann, 2004).

Neutron protein crystallography is an important part of a diverse interwoven history of the broader field of neutrons in structural biology that also includes dramatic developments in many laboratories worldwide (notably BNL, LANL, NIST and ORNL in the USA, ILL in Europe, JAERI in Japan and ISIS in the UK) in small-angle neutron scattering (SANS), low-angle diffraction, reflectometry and inelastic scattering. Many of the early achievements in structural biology using neutron scattering were presented at the Brookhaven Symposium on Neutrons in Biology held in 1975 (Schoenborn, 1976). This symposium was organized at the time when the new reactor at the Institute Laue–Langevin (ILL; Grenoble) started to have an impact. The sophisticated instruments, such as the SANS instrument D11 located on a cold source, immediately generated superb data that enabled the analysis of, for example, chromatin (Baldwin *et al.*, 1975; Hjelm *et al.*, 1977) and protein complexes (see, for example, Stuhrmann, 1974; Marguerie & Stuhrmann, 1976). The development of structural biology at the ILL was in part a consequence of the establishment of the EMBL outstation by Sir John Kendrew under the able scientific leadership of Andrew Miller. With BNL and ILL as two major centers of activity, the worldwide interest in neutrons and structural biology grew substantially

and many of the highlights were presented at the second Brookhaven Symposium on Neutrons in Biology Conference held in 1981 (Schoenborn, 1984) and subsequently the third held at LANL in 1995 (Schoenborn & Knott, 1996). In more recent years there have been numerous conferences and workshops dedicated to various aspects of this very active and productive field of research. There are also excellent reviews and monographs (see, for example, Fanchon *et al.*, 2000; Fitter *et al.*, 2006).

2. The beginning

In the spring of 1965, I had just finished a study on the binding of the anaesthetic Xe to Mb and was trying to calculate the binding energies. There were 32 protein atoms within van der Waals bonding distance of Xe and most were H atoms, the locations of which were based on structural assumptions and were therefore only approximate. Clearly, a map depicting actual H-atom locations was needed. At a teatime discussion at the MRC Laboratory (Cambridge, England), we discussed a number of options, but only neutron diffraction was considered to be a distant possibility. Subsequent discussions with neutron scattering experts at the Atomic Energy Research Establishment (Harwell, England) were not encouraging and I shelved the idea for the time being. During a seminar on the binding of Xe to proteins at the Biochemistry Department in Berkeley, I mentioned the neutron approach and Professor Koshland suggested that I obtain beam time on the High Flux Beam Reactor (HFBR) at Brookhaven National Laboratory (Upton, USA), a laboratory which would play a key role in the early developments. With the help of William Hirs, I obtained a position at BNL and commenced what many considered to be a wild-goose chase.

In mid-1968 I obtained a few days of beam time at Walter Hamilton's single-crystal diffractometer. It was a monochromatic four-circle instrument with a single detector with one of the first computer-controlled systems made by BNL using a Scientific Data Systems (SDS) computer. After a few hours of adjusting the automation we found the first reflection (Fig. 1), which turned out to be the 6 0 3. The crystal was soaked in D_2O to reduce background from the incoherent scattering of neutrons by H atoms. Even with the large 25 mm^3 crystal, an ω–2θ scan step of 0.1°, a 2° reflection scan width and a step time of 1 min, it would take an enormous amount of time to collect even a 2.8 Å resolution map. Over the next year I collected 4800 reflections and produced a Fourier map that demonstrated that H-atom locations could be determined with a 2.8 Å resolution map (Schoenborn, 1969).

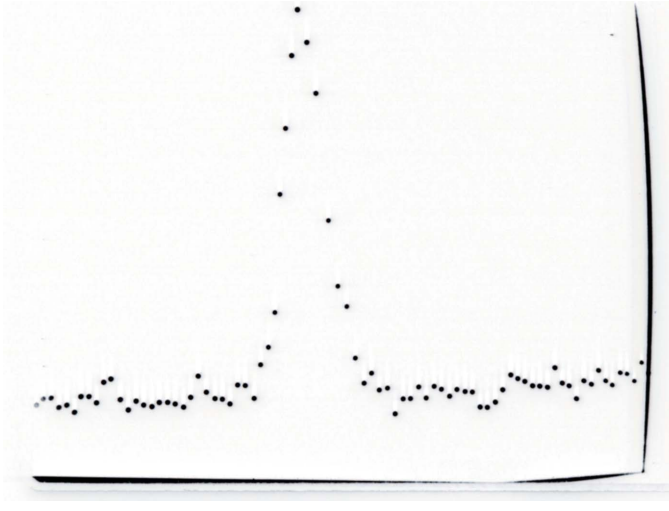

Figure 1
The first reflection (6 0 3) collected from a single crystal of myoglobin using a single-crystal diffractometer on the High Flux Beam Reactor (HFBR) at Brookhaven National Laboratory (BNL). The vertical axis is the scattered intensity and the horizontal axis is the scanning angle.

These first experimental data established beyond doubt that neutron diffraction would provide valuable information on the structure of proteins. They also established that major advances in instrumentation, data collection and data analysis were essential in order to maximize the contribution of the technique. I learned very quickly that large crystals were needed, which was fortunately quite easy for sperm whale Mb; however, the major advances in protein biochemistry which would ultimately lead to the preparation of fully functional perdeuterated proteins were perhaps not quite so obvious at the time. Nevertheless, these advances did come and opened up unprecedented opportunities for high-resolution studies to answer more difficult questions.

3. Neutron protein crystallography

Although we used high-resolution neutron diffraction to reveal H-atom positions (both in the protein and the solvent; Knott & Schoenborn, 1993), we were also able to distinguish nitrogen from carbon or oxygen. This was used to resolve ambiguities in X-ray crystallographic studies, particularly, for example, the orientation of histidines. To reduce background scattering during data collection and enhance the localization of exchangeable H atoms, we exchanged the H_2O solvent in most single crystals with D_2O. This led us immediately to the realization that H_2O/D_2O exchange could (i) provide useful information on protein dynamics and (ii) enhance protein/solvent contrast in diffraction and small-angle neutron scattering (SANS) experiments.

3.1. Advanced instrumentation and data collection

Our first efforts to achieve a position-sensitive detector (PSD) were simply to replace the single detector with a five-detector system using a white beam with individual monochromators (Schoenborn & Nunes, 1972). However, this was soon replaced with an in-house-built linear PSD using a graphite-coated anode. Unfortunately, this device proved to be quite unstable and the Instrumentation Division at BNL then developed a 30 cm long linear ^{3}He PSD. This started a long-term and very productive relationship with Vjelko Radeka and colleagues in the Instrumentation Division. Initially, we were faced with a number of choices for the technology of large and efficient PSDs; however, we consid-

ered that a gas detector, specifically the multi-wire proportional counter (MWPC), would provide the best option for the types of systems envisaged. The major challenges included the detection efficiency and spatial resolution, especially given the large sizes that we were planning. We started with small active areas of 18 × 18 cm; however, we were soon building a 50 × 50 cm high-efficiency detector for the SANS instrument.

Major improvements in both SANS research and in protein crystallography were achieved by use of these efficient PSDs with good resolution and position stability (Schoenborn *et al.*, 1978; Alberi, 1976; Cain *et al.*, 1976; Fischer *et al.*, 1983; Radeka *et al.*, 1996; Schoenborn, 1983*a*). The sophisticated MWPC technology can now produce detectors of incredible performance, such as the detector built for an instrument on the spallation neutron source at LANSCE and the reactor source at ANSTO. This detector is cylindrical and covers 120° 2θ with a radius of 70 cm. The neutron-sensitive area is contiguous with eight separate readout systems to achieve a total counting rate of more than 10^6 neutrons s^{-1}.

Data collection from protein single crystals had a slow start and only with the development of two-dimensional PSDs were we able to collect sufficient data to look at least at a few macromolecules (Schoenborn, 1971; Schoenborn & Hanson, 1980; Phillips & Schoenborn, 1981; Hanson & Schoenborn, 1981; Koeppe & Schoenborn, 1985; Daniels *et al.*, 1996). To enable us to obtain good peak integration, we had to develop new tools to extract the often weak peaks from background (Schoenborn, 1983*b*). A first step in data analysis was the correction of detector counting-rate variations arising from imperfections in the back plane and wire thickness, as well as the radial decrease of the anode potential. This was achieved by a look-up table created through uniform illumination of the detector. To obtain good peak-to-background ratios, particularly for the weak reflections, the reflection-integration scheme used involved the pre-calculation of peak shapes from the known diffraction parameters $\Delta\lambda$, crystal mosaicity, detector resolution and diffraction angle (Schoenborn, 1983*b*). These pre-calculated peak shapes were refined using strong observed reflections and were then used as masks to extract the reflections and delineate the background. A three- or five-element filter system developed by Kossiakoff was used to scan the two-dimensional detector array and was effective in finding large peaks for crystal-orientation and unit-cell parameter refinement. Unfortunately, however, this technique often overestimated weak reflections by picking uneven features in the background. Promising trial data-integration runs using a modified version of *MADNESS* for data integration took place just before the permanent shutdown of the HFBR.

3.2. Data analysis

Protein refinement started with known phases from X-ray studies and used classical refinement approaches such as real-space techniques (Hanson & Schoenborn, 1981; Norvell & Schoenborn, 1976), which were later supplanted by rigorous least-squares programs such as *PROLSQ* and molecular-graphics programs such as *O*. The use of D_2O solvent with a hydrogenous protein requires special treatment of the solvent structure if accurate surface water molecules are to be determined. Another improvement in neutron structure refinement involved the better determination of restraints for H and D bonds (Schoenborn, 1987). These developments have been rewarded with a number of crystallographic studies at BNL of macromolecules including Mb (Phillips & Schoenborn, 1981; Hanson & Schoenborn, 1981; Cheng & Schoenborn, 1990), trypsin (Kossiakoff & Shteyn, 1984; Kossiakoff & Spencer, 1980, 1981), crambin (Teeter, 1984; Teeter & Kossiakoff, 1984) and later cyclosporin (Knott *et al.*, 1988, 1990), plastocyanin (Church, 1992), concanavalin (Gilboa & Yariv, unpublished work), fatty acid-binding protein (Sacchettini & Scapin, unpublished work; see comment in Sacchettini *et al.*, 1992) and others.

4. Protein dynamics

Proteins are not static objects and any description of structure incorporates some element of dynamics. The structure determined by X-ray diffraction techniques will include the thermal motion (and possible disorder) of the individual non-H atoms and a neutron diffraction analysis will provide similar information with additional data on the H atoms. Depending on the resolution of the data, the thermal motion of H atoms will be included in the refinement.

For trypsin, this information was used to resolve an uncertainty in the rotation of terminal methyl groups. Spectroscopic studies had established that methyl groups exhibit rapid rotation, but provided little information on their preferred orientations. It was found from a high-resolution neutron diffraction structure that 85% of the ordered methyl groups were within 20° of the staggered conformation. This suggested that their rotation is quantized in 120° steps about a position of highest stability (Kossiakoff, 1983).

Dynamics in other time domains can be explored by the distribution of exchanged H atoms throughout the protein structure when an unexchanged protein is exposed to D_2O. This distribution is a result of the exchange mechanism(s) on a scale dictated by the solvent-exchange time (which is between weeks and years). Clearly, solvent accessibility plays a dominant role in the mechanism. For example, amide H atoms located on exposed turns of α-helical segments are readily exchanged (Schoenborn, 1972); however, access to some H atoms involves cooperative motions including local breathing and global unfolding (Kossiakoff & Shteyn, 1984; Mason *et al.*, 1984).

5. Protein solvent structure

The detailed analysis of water in Mb solved a long-standing controversy between magnetic resonance studies, which observed only a few bound water molecules, and X-ray structures, which found close to 100 bound water molecules (Otting *et al.*, 1991). The neutron map clearly showed that only three water molecules were bound by three deuterium bonds

and therefore were irrotationally bound to Mb; only these water molecules would show up in magnetic resonance studies. All the other water molecules have some freedom to tumble and exchange, with the O atom more or less in the same location as observed in the X-ray and neutron maps. The neutron maps clearly depicted water molecules with three, two and one deuterium bonds. Such water bonding was further confirmed by molecular-dynamics calculations (Gu & Schoenborn, 1995).

The layers of solvent that surround a protein molecule mediate its functional conformation as well as its biochemical characteristics. We obtained useful information on this interface region from high-resolution neutron diffraction studies since water constitutes approximately 40–60% of the volume of typical protein crystals. Water molecules that are hydrogen/deuterium bonded to the protein surface can be directly visualized as integral components of the structure (Phillips & Schoenborn, 1981; Teeter, 1984; Raghavan & Schoenborn, 1984; Savage & Wlodawer, 1986). Of course, the O atoms of well ordered water molecules are assigned in X-ray structures.

The localization of water molecules is usually correlated with the surface characteristics of the protein. Ordered water molecules have been located in hydrophilic regions and small water clusters have been observed quasi-randomly distributed over the protein surface. We developed a formalism that modelled the solvent as a series of shells with spatial and physical characteristics (Schoenborn, 1988; Cheng & Schoenborn, 1991*a*,*b*). Progressing outward from the protein surface, each shell was constructed of pseudoatoms arranged on a three-dimensional grid. Each pseudoatom was assigned coordinates and a global factor that represented the degree of order (or liquidity) within the shell. The solvent density was then refined by a minimization technique comparing observed low-angle reflections with the calculated density. This resulted in a best solvent density and a smearing (temperature) factor *B*. This approach greatly improved subsequent least-squares calculations. Such calculations clearly showed a higher water density close to polar groups compared with nonpolar regions. It has been shown in studies of Mb and plastocyanin that such solvent refinement enhances surface characteristics and even adjusts side-chain locations. It was subsequently shown that such an approach is equally valid for X-ray data refinement (Jiang & Brünger, 1994; Shu, 1994).

Refining the solvent structure reveals a wealth of chemical information about the molecule, including the geometry of hydrogen bonding, the protonation states of histidines (Fig. 2) and the location and geometry of water molecules at the surface of the protein (Fig. 3). An X-ray and a neutron data set of a carbonmonoxy-Mb crystal were used for such solvent-structure studies (Shu *et al.*, 2000)

6. Protein deuteration

The many H atoms covalently bound to C atoms in proteins contribute significantly to background scattering even for crystals soaked in D_2O mother liquor. Complete perdeutera-

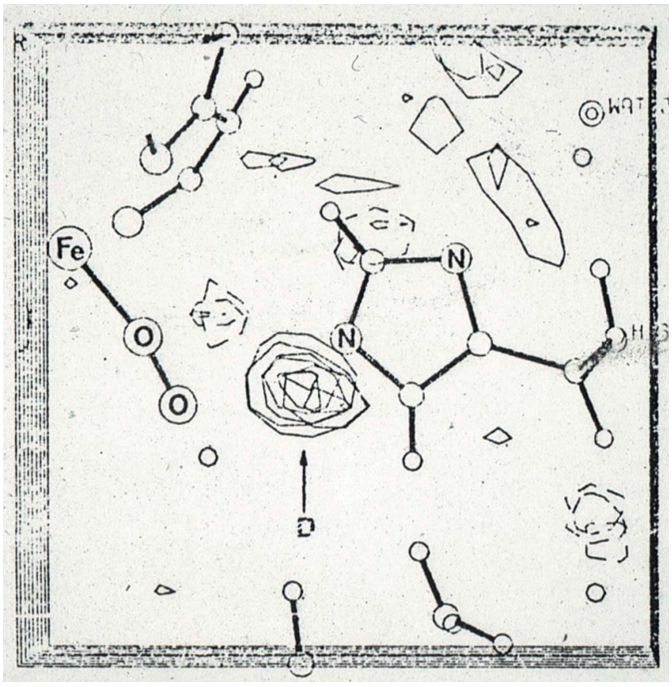

Figure 2
$|F_o| - |F_c|$ neutron difference map in a slab centered on the plane of the imidazole 7E ring in oxymyoglobin. The refined model is superimposed, showing His7E, FeO_2 and part of the heme. The strong positive peak indicates the presence of deuterium bonded to N^{ε}. From Phillips & Schoenborn (1981), reprinted with permission of Macmillan Publishers Ltd, copyright 1981.

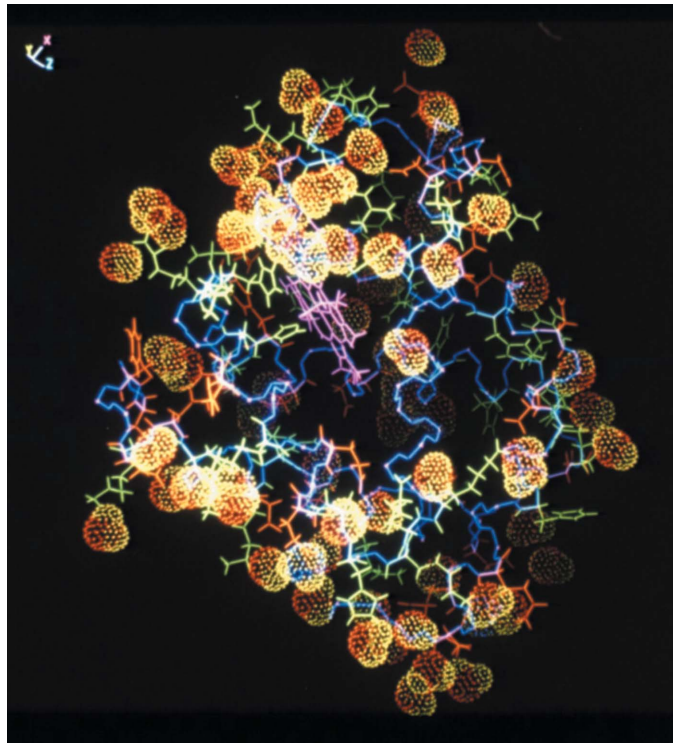

Figure 3
The myoglobin structure with water molecules as determined by neutron diffraction studies. Water is shown as dotted clouds on the surface of the protein. From Cheng & Schoenborn (1990).

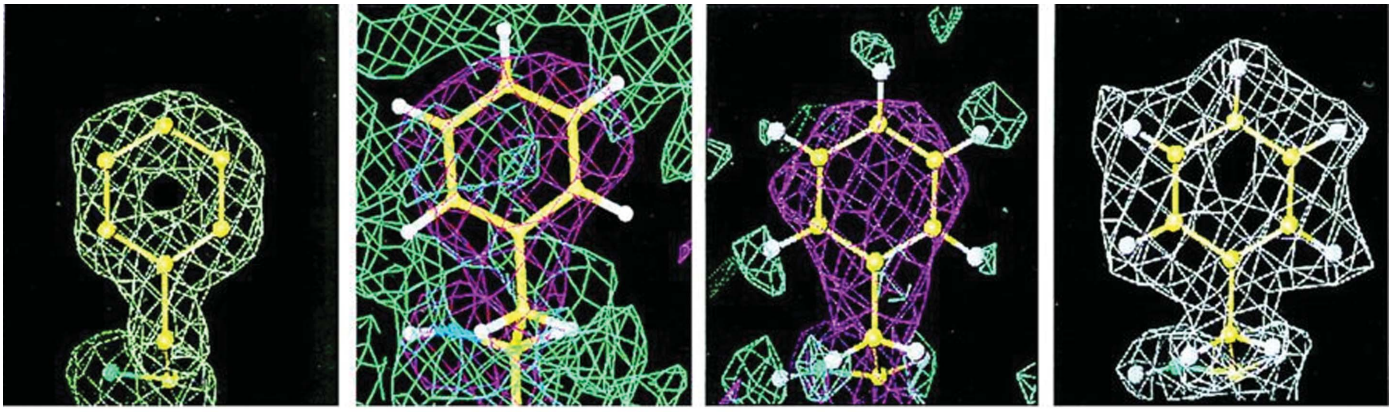

Figure 4
D (H) atoms can be located directly as positive peaks in $2F_o - F_c$ maps, as illustrated by residue Phe43 in oxymyoglobin. (*a*) The $2F_o - F_c$ X-ray map of fully deuterated Mb using 6.0 to 1.5 Å data, contoured at $+1.0\sigma$ (0.84 e^- Å^{-3}). (*b*) A $2F_o - F_c$ neutron map of unlabeled Mb calculated to 2.0 Å resolution, with the pink map contoured at 1.0σ and the blue map contoured at -1.0σ. (*c*) An F_c neutron map generated using equivalent experimental reflections with 6.0–2.0 Å resolution data calculated from the current protein model except that D was replaced with H. The pink map is contoured at $+1.0\sigma$; the blue map is contoured at 2.0σ. (*d*) The $2F_o - F_c$ neutron map of fully deuterated Mb using 6.0–2.0 Å resolution data, contoured at $+1.0\sigma$ (1.03 fm Å^{-3}). After Shu *et al.* (2000). Copyright (2000) National Academy of Sciences, USA

tion of a protein would greatly improve the peak-to-background ratio and subsequently produce more accurate structures, as demonstrated by a study of perdeuterated Mb (Shu *et al.*, 1996, 2000).

Beside the high background arising from the incoherent scattering from H, the negative coherent scattering of H atoms tends to cancel out the positive contribution from other atoms in a neutron-density map. Therefore, a fully deuterated sample would yield better diffraction data with stronger density in the H (in fact D) atom positions. On this basis, a sperm whale Mb gene modified to include part of the λ cII protein gene was cloned into the T7 expression system (Shu *et al.*, 1996). Milligram quantities of fully deuterated holo Mb were obtained and used for crystallization. The synthetic sperm whale Mb crystallized in the $P2_1$ space group isomorphous with the native protein crystal. A complete X-ray diffraction data set at 1.5 Å resolution was collected first and was followed by a 2 Å resolution neutron diffraction analysis. This analysis produced much better data and a vastly improved Fourier map compared with the hydrogenous Mb structure (Shu *et al.*, 2000). The decrease in background scattering alone makes it worthwhile perdeuterating proteins for neutron diffraction studies (Fig. 4). Indeed, macromolecular deuteration laboratories are now integrated into the major neutron scattering facilities worldwide.

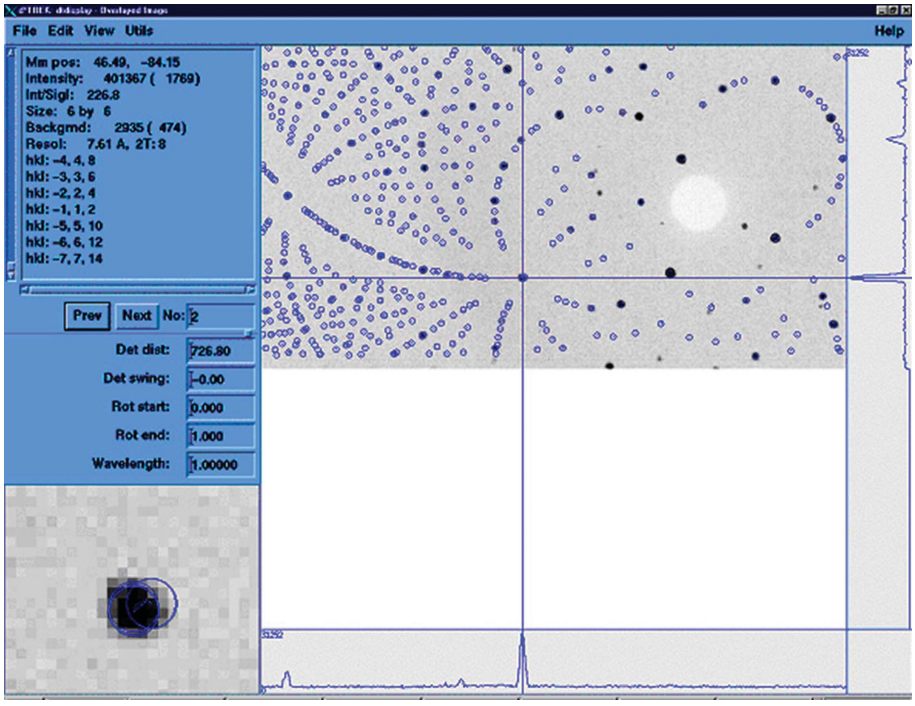

Figure 5
A Laue projection of data collected from D-xylose isomerase on the Protein Crystallography Station (LANSCE) using one crystal setting. A total of 181 797 reflections were recorded over 23 crystal settings (courtesy of Gerry Bunick and Leif Hanson, ORNL).

7. The Protein Crystallography Station at a spallation source

To enhance neutron beam intensity, the quasi-Laue technique (Schoenborn, 1992) is particularly well suited to protein crystallography at reactor sources (see, for example, Niimura *et al.*, 1997) and can be tailored to spallation neutron sources. Classical spallation neutron techniques use fully decoupled moderators producing neutron beams

that travel along beam pipes as a function of their velocity, with short-wavelength neutrons arriving at a target station first, followed by the longer wavelength neutrons. Spallation neutron beams at a given time are nearly monochromatic and over the pulse time produce a contiguous wavelength band typically in the range 0.5–6 Å. The delta function-like (per time slice) characteristics of such fully decoupled moderators can be broadened by using partially decoupled moderators, giving a finite $\Delta\lambda$ but with a fourfold flux increase (Schoenborn *et al.*, 1999).

The Protein Crystallography Station built at the Los Alamos Neutron Science Center (LANSCE) uses such a decoupled moderator. The moderated neutrons are extracted down a beam pipe. From the moderator, neutrons travel a total flight-path length of 28 m down a vacuum pipe with collimation inserts that taper the neutrons to produce a beam with 0.1° divergence (matched to the mosaicity of an average crystal). This 28 m source-to-target length allows observation of neutrons between 0.7 and 6 Å with a $\Delta\lambda$ of about 0.1 Å. A chopper system removes unwanted high-energy and low-energy neutrons to protect the sample and detector from fast neutrons and γ radiation produced during the initial proton pulse. A kappa-circle goniometer allows crystal orientation. A complete data set can consist of many thousands of reflections and typically requires between 12 and 30 crystal settings depending on the symmetry of the crystal (Fig. 5). A large cylindrical PSD fabricated by the Instrumentation Division at BNL collects as many of the spots as possible at each crystal setting without having to reposition the detector. The whole data-collection process involves a number of dedicated computer systems to decode the position of diffracted neutrons, time-stamp the arrival time of the neutron and ultimately integrate and store the reflections (Langan & Greene, 2004). Initial experiments surpassed our expectations and results for D-xylose isomerase, for example, are described elsewhere in this issue.

8. Summary

The foundations of modern structural biology were laid in the 1960s by studies such as those of myoglobin and hemoglobin by John Kendrew and Max Perutz. These pioneering efforts gave us the first insight into the complex relationship between the structure and the function of proteins and raised a myriad of questions about detailed molecular interactions involving basic structural motifs such as hydrogen bonding, charge transfer and nonbonding (van der Waals) interactions. It was clear that detailed analysis of these interactions would require neutron scattering techniques, since imaging H atoms by X-ray protein crystallography is problematic. The 1970s and 1980s were periods of initial major developments: the techniques are now mature and are poised to make a significant and fundamental contribution to structural biology.

I would like to acknowledge all my students, postdoctoral fellows and colleagues who have worked tirelessly over the years to help achieve the goals I set early in my career. I would also like to acknowledge the funding agencies which have contributed to the many activities, especially the US Department of Energy and the National Science Foundation.

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