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19. OTHER EXPERIMENTAL TECHNIQUES

19.1. Neutron crystallography: methods and information content

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19.1.1. Introduction

Neutron and X-ray crystallography are similar in both their experimental methodologies and in the resulting information content. The principal difference between the two methods is brought about by the characteristic scattering potential of the atom types. The scattering of neutrons by material is not proportional to the atomic number, as is the case in X-ray scattering, but rather depends on the individual nuclear characteristics of each atom type. As seen in Table 19.1.1.1, these characteristics show considerably less deviation and systematic trend among the different atom types. For instance, the heavy atoms in biological material – carbon, oxygen and nitrogen – scatter with about the same magnitude as a lead or uranium atom. In addition, neutrons are scattered by the atomic nuclei, which are essentially point sources, producing diffracted intensity not attenuated by a form-factor fall-off at increasingly higher scattering angles, as is the case in X-ray diffraction (Bacon, 1975).

There are a few atomic nuclei that induce a phase change of 180° in the scattered neutron, which results in negative peaks in a neutron density map. An extremely important example of this is the hydrogen nucleus, with a scattering length of -3.7 f ($1\ \text{f} = 10^{-13}$ cm). Its isotope, deuterium, on the other hand, scatters to give positive peaks (+6.7 f). The fact that H and D atoms can be so clearly distinguished from one another has very important implications for assessing biophysical parameters, as will be discussed below.

The application of the neutron-diffraction technique, which assigns H-atom positions in proteins and differentiates between H and D atoms, has been mainly focused on structural issues in three research areas: (1) protein reaction mechanisms; (2) protein dynamics; and (3) protein–water interactions (Kossiakoff, 1985, and references therein). It must be pointed out that recent advances in nuclear magnetic resonance have made protein dynamics investigations using H/D exchange procedures much easier than similar experiments by neutron diffraction. Additionally, the advances in ultra-high-resolution X-ray crystallography, which have allowed some level of experimental determination of hydrogen atoms in proteins, have further limited the uniqueness of the neutron method. Nevertheless, a number of important structural issues that are best approached by neutron crystallography remain.

Table 19.1.1.1. *Scattering lengths for atom types*

Element	Atomic No.	Scattering length (f; $1\ \text{f} = 10^{-13}$ cm)
H	1	−3.7
D	1	6.7
C	6	6.6
N	7	9.4
O	8	5.8
Mg	12	5.2
S	16	2.8
Ca	20	4.7
Hg	80	12.7
Pb	82	9.4
U	92	8.5

19.1.2. Diffraction geometries

The general experimental setup involves use of a monochromated beam, employing normal-beam (Caine *et al.*, 1976) or flat-cone geometry (Prince *et al.*, 1978). Both approaches use flat detector surfaces, and thus there is a distortion inherent in all the diffraction phenomena that increases as a function of layer line along the axis of rotation. The extent of this effect can be calculated from the experimental parameters, but, in the case of a linear detector, there is only a moderate amount of flexibility available to make the necessary adjustments. The flat-cone geometry is well suited for a linear detector, since upper-level data fall on an undistorted plane. However, such a scheme requires that the detector be adjusted to different orientations with respect to the spectrometer axis (Prince *et al.*, 1978). In the normal-beam configuration, the crystal is usually mounted on a four-circle goniometer, allowing independent rotations around the φ, χ and ω axes to cover a full sphere of reciprocal space. This method can be efficient when used with a two-dimensional area detector because of the distortion of the diffraction pattern.

19.1.2.1. *Quasi-Laue diffractometry*

A significant advance in neutron crystallography has been the development and use of modified Laue methods to collect data (Wilkinson & Lehmann, 1991; Wilkinson *et al.*, 1992; Niimura *et al.*, 1997). These methods greatly increase the available neutron flux by using the white neutron spectrum. The full white radiation cannot be used due to very high background scattering and overlap between the diffraction peaks. A reasonable compromise between maximizing intensity while minimizing the experimental problems is to limit the white radiation component to about a 20% wavelength band by employing Ti–Ni multiple-spacing multilayers (Niimura *et al.*, 1997). In practice, the use of the Laue method in X-ray diffraction allows most of the reciprocal space to be recorded in one crystal setting. The quasi-Laue application requires several settings, depending on the neutron intensity distribution $I(\lambda)$ and the crystal symmetry. Data processing can be done using Laue software modified for neutron data.

19.1.3. Neutron density maps – information content

Fig. 19.1.3.1 illustrates several types of structural information derived from neutron density maps. Fig. 19.1.3.1(*a*) shows a well ordered tyrosine ring in the 1.4 Å structure of the protein crambin (Teeter & Kossiakoff, 1984). It can be seen that the ring hydrogen-atom locations are in positions of negative density. These peaks appear to be slightly displaced from their true positions, because the map is not at atomic resolution. At 1.4 Å, a portion of the negative peak of the hydrogen overlaps the positive peak of the ring carbon, effectively cancelling density between the atoms and giving the

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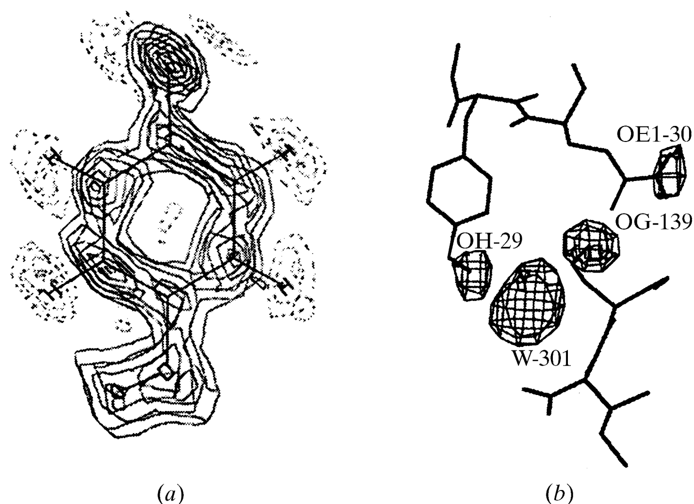

Fig. 19.1.3.1. Information content in neutron density maps. (*a*) A well ordered tyrosine ring in the 1.4 Å refined structure of crambin (Teeter & Kossiakoff, 1984). (*b*) $D_2O - H_2O$ difference density map of a hydrogen-bonding network in trypsin: Gln30 Oε1–Ser139 D_γ, Ser139 O_γ–W301, W301–Tyr29 DO. Water density and H/D exchange density shown.

illusion that the peak has been translated. The hydroxyl deuterium orientation is readily determined by its position in positive density.

Use of $D_2O - H_2O$ neutron difference maps provides a high level of stereochemical information (see below) (Kossiakoff *et al.*, 1992; Shpungin & Kossiakoff, 1986). Fig. 19.1.3.1(*b*) displays a network of three hydrogen bonds involving three side-chain types and an occluded water. With knowledge of the heavy atoms alone, it is not possible to define the donor/acceptor character of any of the side chains, because they can act in either capacity, as can the water. The assignments can be made unambiguously from the $D_2O - H_2O$ density, as can the orientation of the water molecule. These maps have allowed detailed analysis of hydroxyl orientations in protein molecules (Kossiakoff *et al.*, 1990, McDowell & Kossiakoff, 1995).

Neutron diffraction is an ideal method for investigating methyl-group conformation, because it allows direct observation of hydrogen-atom positions (Fig. 19.1.3.2) (Kossiakoff & Shteyn, 1984). Although methyl groups in proteins are not held in fixed positions, but spin rapidly around their rotor axes, the time-averaged character of the diffraction experiment establishes the low-energy conformer and the degree of disorder. Accurate methyl-group analysis requires relatively higher resolution (1.5 Å or better) than characterizing other structural features.

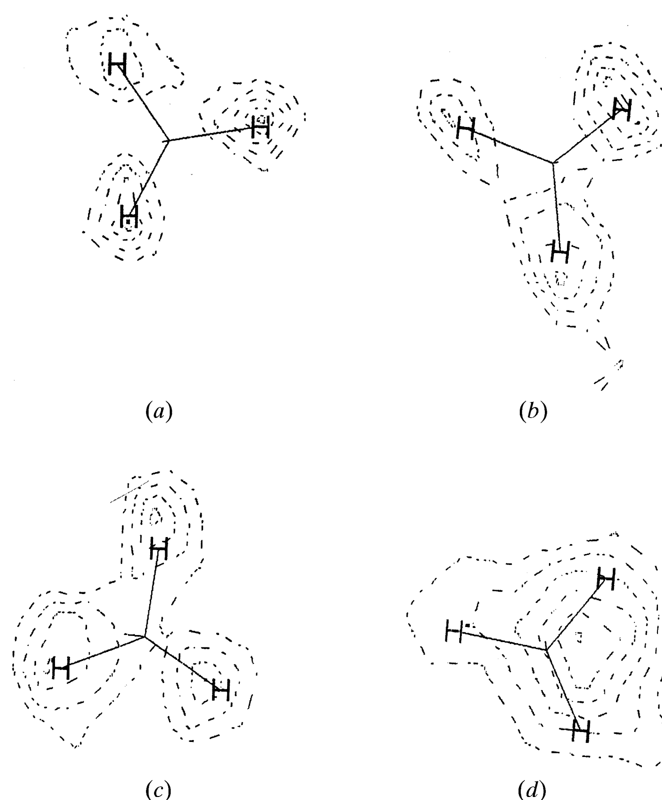

Fig. 19.1.3.2. Sections of a neutron difference Fourier map showing methyl hydrogen densities for several representative methyl groups. No phasing information about the methyl hydrogens was included in the model; therefore, hydrogens should appear in the difference map at their true positions but at reduced density ($\sim$ half weight). The groups shown are: (*a*) Ala24, (*b*) Thr21, (*c*) Thr28, and (*d*) Ala45.

19.1.4. Phasing models and evaluation of correctness

Neutron diffraction does not lend itself to the multiple isomorphous phasing approach. This is because the range in atomic scattering power is much narrower than for the X-ray case. There are a few relatively rare isotopes where a significant anomalous effect exists; however, they are not adequate for getting primary phasing information (Schoenborn, 1975). In practice, the initial phasing model has to be derived from the X-ray-determined structure. This is done by applying the appropriate neutron scattering lengths to the refined X-ray coordinates (Norvell & Schoenborn, 1976). Thus, at least in the early stages of analysis, the neutron model relies heavily on the accuracy of the X-ray structure. The importance of an accurate phasing model is borne out by the fact that in several investigations the phasing models were not accurate enough to allow the structure to be refined successfully.

19.1.5. Evaluation of correctness

It is an important first step in the structural analysis to determine the quality of the phases derived from the X-ray structure (Kossiakoff, 1983). Several methods have been used. Using the initial phasing model, the most powerful tests examine an unbiased neutron Fourier map for the appearance of features that are independent of the model. The presence or absence of these features, especially those resulting from the scattering of hydrogen and deuterium atoms, is the most reliable measure of the phasing model. One such test is to evaluate the appearance of the water structure, *i.e.*, the water molecules hydrogen-bonded to the surface of the protein. The water molecules observed in the X-ray analysis are excluded from the neutron-phasing model. The test is applied in cases where the crystals have been soaked in D_2O. The peaks in the neutron density map that correspond to the strongly coordinated water-molecule positions owe their existence solely to the neutron data and phasing model. Even at an early stage, because of the large neutron-scattering potential of D_2O, many of these tightly bound waters found in the X-ray structures should also be observable in the neutron density map.

Another aspect to test phasing reliability is the ability to identify the orientation of side-chain amide groups of asparagine and glutamine. The difference in neutron scattering between O and the two deuteriums and the Nδ2 (5.8 f *versus* 22.6 f) is large enough to be detectable in the Fourier map when these groups are well ordered (Fig. 19.1.5.1). The use of unexchangeable hydrogens for evaluation is considerably more complicated, despite the fact that they constitute about one-half the total number of atoms in the molecule. The difficulty arises from the negative scattering character of the hydrogens, which displaces their apparent positions

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