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Neutron Protein Crystallography in JAERI

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A high resolution neutron diffractometer, BIX-3 dedicated to protein crystallography has been successfully constructed in JRR-3M, JAERI. With this BIX-3 single crystal structure analyses of rubredoxin and myoglobin have been carried out in 1.5 Å resolution which is firstly realized in neutron protein crystallography. The detailed structural informations of hydrogen bonds and H/D exchange have been clearly obtained in the proteins.

KEYWORDS: protein crystallography, neutron diffractometer, high resolution data, hydrogen bond, H/D exchange

§1. Introduction

The three dimensional structure determinations of biological macromolecules such as proteins and DNA by X-ray single crystal structure analysis has helped shed some light on many of the mysteries involved in life processes. At the same time, these results have clearly suggested that hydrogen atoms and water molecules around proteins and DNA play a very important role in many physiological functions. However, since it is very hard to determine the positions of hydrogen atoms in protein molecules using X-rays alone, a detailed discussion of protonation and hydration sites can only be speculated upon so far. In contrast, it is very well-known that neutron diffraction provides an experimental method of directly locating hydrogen atoms. In order to carry out this research in Japan Atomic Energy Research Institute (JAERI), two kinds of diffractometers dedicated for protein crystallography have been constructed where technical innovations such as a neutron imaging plate[1] and a monochromator[2] had to be developed. By using these diffractometer, neutron diffraction experiments from rubredoxin, myoglobin and hen egg-white lysozyme have been carried out and the several results of these proteins are reported in the paper.

In neutron diffraction experiments proteins are crystallized or soaked in D_2O solution in order to avoid very strong incoherent neutron scattering from hydrogen atoms of H_2O in a crystal, and hydrogen atoms attached to N or O are exchanged by deuterium atoms in most cases. Therefore, in this paper hydrogen (H) means deuterium (D) even though hydrogen and deuterium might be mixed to use.

§2. BIX-2 and BIX-3

Laue diffractometry[3] is useful for quick data collection, but suffer from an upper limit of 2 Å in resolution because of background problems. On the other hand, the fixed crystal method (using a monochromatized neutron beam and an area detector) provides the best S/N ratio, although this technique needs a large amount of computer storage since the sample crystal rotates at a small step angle and many frames of data collected at various positions need to be stored in memory. More importantly, this method is more time-consuming than the Laue diffractometry. However, especially in neutron protein crystallography, the most important thing is to collect a data set under the best S/N conditions since the neutron diffraction technique virtually guarantees the detection of all the hydrogen atom positions in proteins once data have been collected. Thus, the monochromatized beam utilization technique is expected to be the method of choice for collecting high resolution data (1.5 Å or better) although it takes much machine time.

Monochromatized neutron beams represent the oldest and most classical sources of neutrons, and most of the previous neutron protein crystallographic data sets have been collected using this type of radiation. Monochromatized neutron utilization is still charming in its way as discussed in comparison with Laue methods. In this method, a monochromator is indispensable in order to obtain a neutron beam of a specific wavelength from a 'white radiation' source. At JAERI, a new type of elastically-bent perfect-Si crystal (EBP-Si) monochromator for a neutron diffractometer, dedicated to protein crystallography, has been developed.[2] The EBP-Si monochromator has been successfully applied to two diffractometers named as BIX-2[4] and BIX-3[5] at JAERI. The detail of the BIX-3 is introduced in the symposium.[6]

§3. Rubredoxin

With this BIX-3 a single-crystal neutron diffraction analysis of the structure of small protein rubredoxin from the hyperthermophile *Pyrococcus furiosus* is currently under way.[7] Rubredoxins are small protein containing an iron atom coordinated by sulfur atoms of four cysteine side chains. Although the physiological role for rubredoxins have not been definitively established, it is likely that they function as electron transfer proteins. Despite the uncertainty of its function in most species, rubredoxins from different organisms have been extensively studied by structural and spectroscopic methodologies since rubredoxin from the hyperthermophile *Pyrococcus furiosus* is extraordinary stable in heat. In order to understand the stability, very high resolution X-ray

crystallography study has been carried out by comparing the structure between wild and the mutant which is not so stable in heat, but the special differences of backbone structure between them could not been found.[8] We assume that there might be differences in hydrogen bonding and/or hydration structures and as a first step a neutron diffraction study of the wild type of rubredoxin with BIX-3. Data were collected at room temperature up to a resolution of 1.5 Å (so far the highest resolution neutron data set), using wavelength λ=2.35 Å. A single crystal of dimension 2 × 2 × 1 mm, grown via vapor diffusion from 3.6 M NaK phosphate, is being used in this project. Two sets of data from the same crystal, roughly corresponding to the crystal being mounted along the a and c axes, are being collected and merged. Data collection is carried out by the step-scan method, with 0.3 deg intervals in ϕ and exposure times ranging from 60 to 75 minutes per frame. The completeness factor of the 1.5 Å resolution data set is currently at 78.2 %. 301 hydrogen atoms and 49 deuterium atoms are included in the refinement of the structure of rubredoxin. 26 water molecules are also identified. In the present model R factor and R-free are 0.254 and 0.279, respectively.

Figure 1 shows the $|2|F_O| - |F_C||$ map of Trp 3. Because of the high resolution, the clear hole is seen in the center of the 6 and 5 members ring and hydrogen atoms and replaced deuterium atom are clearly seen. The replacement ratio of hydrogen atoms by the deuterium atoms bound to nitrogen, oxygen and sulfur atoms has been determined and it is strongly correlated on ASA (Accessible Surface Area) and the temperature factors. The results are shown in Fig.2. The detail of the refined structure is reported in the symposium.[7]

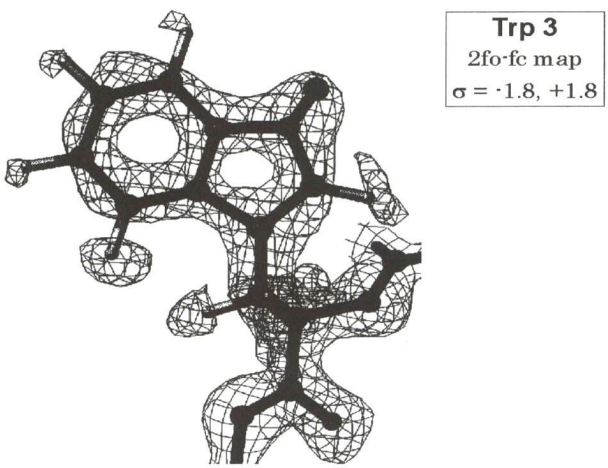

Fig.1. The $2|F_O| - |F_C|$ map of Trp 3 in rubredoxin.

§4. Myoglobin

Myoglobin serves as a "model-protein" in biophysics. It is investigated by many research groups with a wide range of physical and biochemical methods. The respiratory heme protein myoglobin from sperm whale which we have used for the neutron diffraction experiments consists of 153 amino acids which form 8 α-helices. We determined the neutron structure of met-myoglobin up to 1.5 Å resolution. The myoglobin crystal (2.5 × 2.5 × 1.0 mm^3) used in this study was soaked in deuterated mother liquor for years.[9] The space group is $P2_1$ with the lattice constants a = 64.8 Å, b = 31.1 Å, c = 35.0 Å and β = 105.8 deg. Neutron diffraction data were collected at room temperature at the neutron single crystal diffractometer BIX-3. This high resolution data set provides the possibility to analyze hydrogen bridges as well as the extent of exchange of hydrogen atoms by deuterium within the protein.

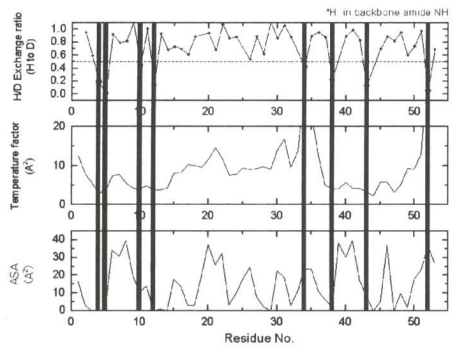

Fig.2. The replacement ratio, ASA and the temperature factor of hydrogen atoms by the deuterium atoms bound to nitrogen, oxygen and sulfur atoms in rubredoxin.

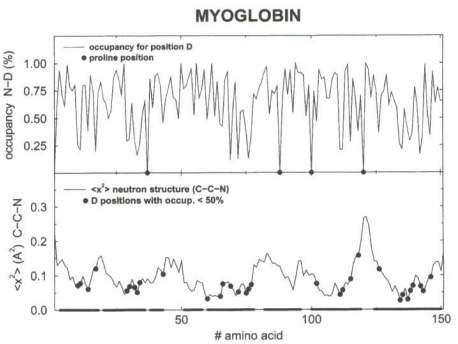

Fig.3. The replacement ratio and mean displacement of hydrogen atoms by the deuterium atoms bound to nitrogen in myoglobin.

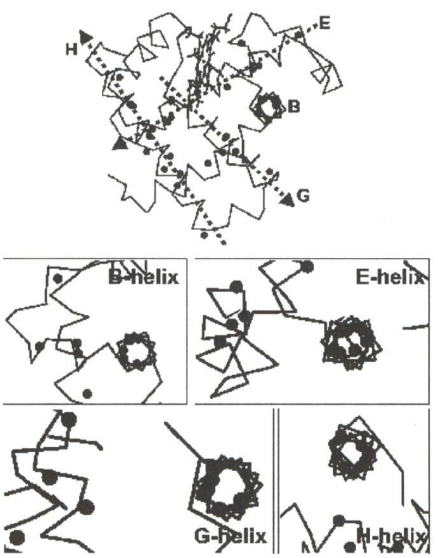

Fig.4. The location of deuterium atoms which has the replacement ratio less than 50 % in myoglobin.

The occupancy of deuterium atoms which corresponds to replacement ratio of hydrogen atoms by the deuterium atoms bound to amide nitrogen atoms has been determined and it is strongly correlated on the local position of amide atoms and the temperature factors as shown in Fig.3 and 4. Figure 3 shows the occupancy and mean displacement of atoms of deuterium atoms of amide, and Figure 4 shows the location of deuterium atoms with the occupancy less than 50 %, and it is clearly localized in the hydrophobic region.

§5. Hydrogen bonding in α-helix

The α-helix in proteins is one of the fundamental secondary structure. It is famous that the existence of the helix structure model proposed by Pauling was firstly verified by the X-ray crystal structure analysis of myoglobin. The helix structure has been stabilized by the formation of straight N-H···O hydrogen bonds between N-H group in the amino acid residue n and C-O group in residue n+4 as originally proposed by Pauling. However, the formation of hydrogen bonds has been characterized by the discussion of the distance between proton donor and acceptor and precise structure of hydrogen bonds including hydrogen atoms has remained in dispute, simply because it is difficult for X-ray crystallography to identify all the hydrogen atoms in proteins.

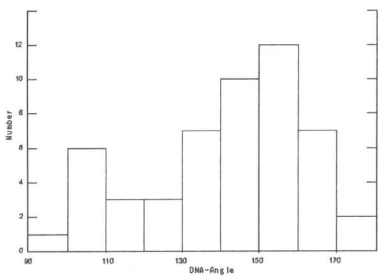

Fig.5. The angle distribution of hydrogen bonds (N-D···O angles) in the α-helix of HEWL.

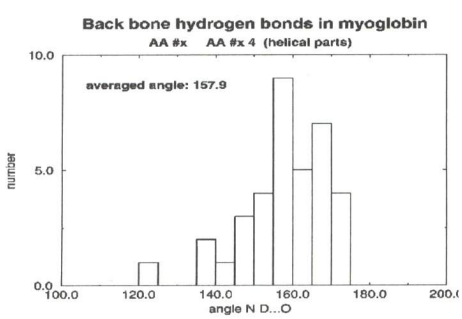

Fig.6. The angle distribution of hydrogen bonds (N-D···O angles) in the α-helix of myoglobin.

On the other hand, neutron diffraction provides an experimental method of directly locating hydrogen atoms and the nature of hydrogen bonds. Neutron diffraction data from tetragonal hen egg-white lysozyme (HEWL)[10] and myoglobin[9] were analyzed and the position of all the hydrogen atoms in both of the protein were identified.

The identification of hydrogen atoms provides us the

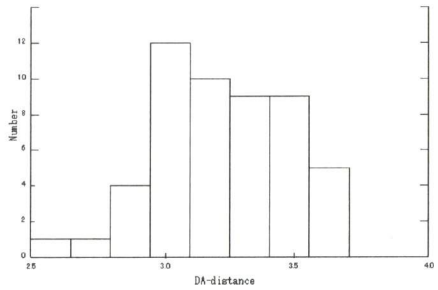

Fig.7. The distance distribution of hydrogen bonds (N···O) in the α-helix of HEWL.

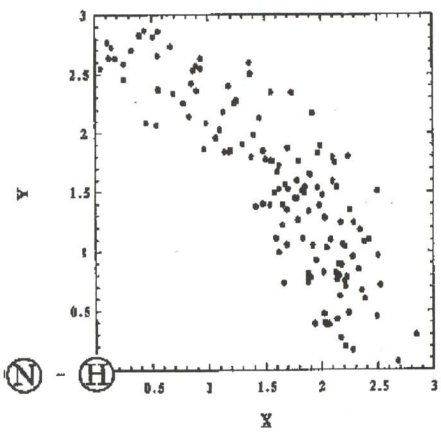

Fig.8. The distribution of oxygen atoms in the N-H···O arrangement when the N-H bond lies on the horizontal axis as H atom is fixed on the origin.

accurate information of hydrogen bonds in proteins. There are 6 α-helices in a HEWL molecule and 8 α-helices in a myoglobin. The "N-D···O" type intramolecular hydrogen bonds in the α-helices, which play an important role in the stabilization of the α-helices of proteins, were surveyed.

Figure 5 shows the angle distribution of hydrogen bonds (N-D···O angles) in the α-helix of HEWL. According to the Pailing's model, N-D···O angles should be 180 deg since N-D···O bonding is assumed to be straight, but Fig. 5 clearly shows that there are no straight hydrogen bonds. It is confirmed in myoglobin, too. Figure 6 shows the angle distribution of hydrogen bonds (N-D···O angles) in the α-helix of myoglobin.

Figure 7 shows the distance distribution of hydrogen bonds (N···O) in the α-helix of HEWL. Generally the formation of a hydrogen bond is defined when N···O distance is less than 3.6 Å and when the distance is extraordinary short, it might be discussed that there forms a strong hydrogen bonding. As a matter of fact, there is an example that the N···O distance is very short, such as 2.6 Å as shown in Fig.7 but it was found that the H···O distance in the N-H···O arrangement is normal as shown in Fig.8. Figure 8 shows the distribution of oxygen atoms in the N-H···O arrangement when the H atom in the N-H is fixed on the origin. It is seen that the oxygen atom

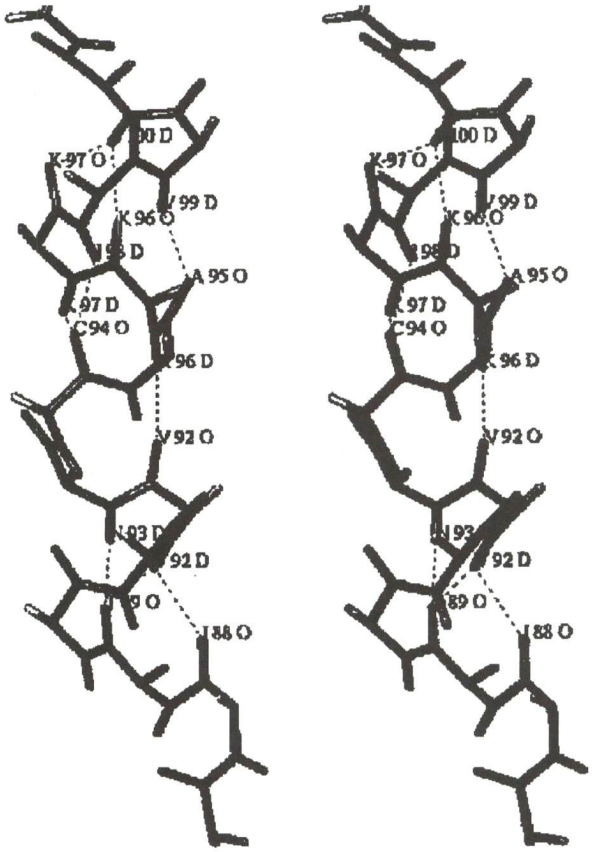

Fig. 9. A stereo view of the bifurcated hydrogen bonds in HEWL.

position scatters while the H···O distance is kept to be almost constant. The N···O distance becomes very short apparently since the N-H···O arrangement bends.

Because of the bent N-H···O arrangement, the bifurcated or trifurcated hydrogen bonds were found. Figure 9 shows an example of the bifurcated hydrogen bonds.

To study the nature of these uncommon hydrogen bonds, it is essential to have positional information about the hydrogen atoms.

§6. Conclusion

It was absolutely proved that high resolution neutron protein crystallography provides us the accurate knowledge of hydrogen atoms. We have just started to understand the real structural nature of hydrogen bonds and H/D exchange where hydrogen atoms take part in. The next step will be hydration where orientation and coordination of water molecule become important and the identification of hydrogen atoms in water molecules is indispensable. High resolution protein crystallography will contribute to the field.

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