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Neutron Diffraction Study on the Structure of Rubredoxin from *Pyrococcus furiosus*

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With the new single-crystal diffractometer BIX-3 at the JRR-3M reactor of JAERI, a single-crystal neutron diffraction analysis of the structure of the small protein rubredoxin from the hyperthermophile *Pyrococcus furiosus* is currently under way. Data were collected at room temperature up to a resolution of 1.5 Å (the highest resolution obtained thus far for a neutron data set). Data collection was by the step-scan method, with 0.3° intervals in ϕ and exposure times ranging from 60 to 77 minutes per frame. The completeness factor of the 1.5-Å resolution data set is currently at 76.8 %. Included in the refinement are 301 hydrogen atoms and 40 deuterium atoms, and 29 water molecules were also identified. In the present model, the current value for R and R_{free} are 24.0 % and 26.3 %, respectively.

KEYWORDS: neutron crystallography, structural biology, rubredoxin, *Pyrococcus furiosus*, BIX-3

§1. Introduction

About half of the atoms in a protein molecule are hydrogen atoms, and a protein is always surrounded by water molecules of hydration. It is beyond doubt that they play an important role in biological systems. They are indispensable substances in many naturally-occuring processes (e.g. hydrolysis, dehydrogenation, oxidation-reduction, and so on). In addition, H atoms are intimately involved in the folding and stabilization of protein structure through hydrogen bonds. Therefore, the determination of accurate positions of hydrogen atoms of a protein and its hydration water molecules would be useful in understanding the mechanism, function, and stability of many important proteins.

X-ray crystallography is a powerful method for the analysis of macromolecular structures. However, it does not provide all the desired structural information because it is usually difficult in an X-ray analysis to locate the positions of hydrogen atoms. In contrast, neutron diffraction can easily visualize hydrogen positions. By using this method, the details of hydrogen bonds can be directly determined, whereas in the X-ray method the locations of the H atoms can only be inferred from the positions of the C, N and O atoms of the molecule. The orientation of hydration water molecules may also be determined in a neutron study. In addition, neutron diffraction can decide whether H/D exchange has occurred at a particular site, because H and D have dramatically different neutron-scattering properties. Thus, X-ray and neutron diffraction methods are complementary, and between the two techniques it is possible in principle to determine all of the atomic positions in a protein.

The number of neutron studies of biomacromolecules reported up to now is not large because of the low flux of neutrons from even the best sources, which leads to long data collection times. In order to overcome this disadvantage, we have developed a neutron imaging plate (NIP)[1)] which is the key component in a large area detector system now available. It drastically reduces data collection times because it enables a large number of Bragg reflections to be recorded simultaneously and relatively quickly. A few studies using NIP detectors in neutron crystallography have been reported so far.[2–4)] Recently we have developed a new NIP-based diffractometer called BIX-3 at the JRR-3M reactor of JAERI. The architecture and performance of BIX-3 is discussed elsewhere.[5,6)] The present study on rubredoxin is the first one performed using this new diffractometer.

Rubredoxins are small proteins containing an iron atom coordinated by the sulfur atoms of four cysteine side chains. Although the physiological role of rubredoxins has not been definitively established, they almost certainly function as electron transfer proteins, possibly in oxygen detoxification.[7)] Owing to their small size, stability, and ease of isolation, rubredoxins from different organisms have been extensively studied by numerous structural and spectroscopic methods.

X-ray diffraction analyses of the structure of rubredoxin from the hyperthermophile *Pyrococcus furiosus* (at 0.95 Å resolution) and two of its mutants (at 1.1 and 1.2 Å resolution, respectively) have previously been conducted by some of us in an attempt to understand the differences in thermostability between the different variants.[8)] In that X-ray study, about half of hydrogen atoms of the wild-type rubredoxin could be clearly seen in the difference maps because of the high resolution of the data. However, no simple correlation between the structures of these proteins and their thermostabilities could be found.

The present investigation is motivated by the hope

Table I. Data-collection and refinement statistics

Data collection	
Space group	$P2_12_12_1$
Unit-cell parameters a, b, c (Å)	34.32, 35.31, 44.23
Maximum resolution (Å)	1.5
Number of observed reflections	16201
Number of unique reflections	6995
Completeness (%)	76.8 (52.9)*)
R_{merge} (%)	9.7 (24.1)*)
$I/I(\sigma)$	7.5 (3.0)*)
Crystallographic refinement	
Resolution range (Å)	5-1.5
R value with $F > 2\sigma_F$ cutoff (%)	24.0
R free with $F > 2\sigma_F$ cutoff (%)	26.3
Number of hydration water molecules	29
Number of identified hydrogen atoms	301
Number of identified deuterium atoms	40

*) Numbers in parentheses are for the highest resolution shells (1.50-1.55 Å).

that the differences in thermostability might be explained using the more accurate parameters of hydrogen bonds obtained by neutron crystallography. The present neutron diffraction study on wild-type rubredoxin is the starting point for a structural comparison with its mutants which have different thermostabilities.

§2. Methods

2.1 Crystallization

The construction of the rubredoxin gene, its overexpression in *E. coli*, and the isolation of rubredoxin are described elsewhere.[9] Crystals of wild-type rubredoxin were grown by the sitting-drop vapour diffusion method coupled with micro-seeding techniques. A single crystal having dimensions of 2.5 × 2.5 × 0.8 mm was mounted in a sealed quartz capillary of diameter 3 mm. The rubredoxin solution used in the crystallization experiments contained 40 mg ml^{-1} protein, 50 mM Tris/Tris-HCl buffer (pH 8.0), and 0.3 M NaCl. The reservoir contained 3.8 M NaK phosphate (equimolar NaH_2PO_4 + K_2HPO_4) as precipitant. These conditions are similar to those described earlier.[8] In order to reduce background scattering from H atoms, which have a large incoherent scattering cross section, the rubredoxin solution was subjected to H_2O/D_2O exchange prior to crystallization. This allows D to be substituted for H, not only in the water molecules of hydration, but also at sites containing "exchangeable" hydrogen atoms (mostly H atoms in N-H and O-H bonds exposed to the solvent). Such protons have previously been described for this protein.[10]

2.2 Data collection

Data were collected at room temperature on the BIX-3 diffractmeter[5,6] installed on the 1G-A site of the JRR-3M reactor at JAERI. An elastically-bent perfect-Si crystal (EBP-Si) monochromator[11,12] was used and the wavelength was 2.35 Å. Two sets of data from the same crystal, corresponding to the crystal being mounted along the a and c axes, (resolution 1.5 Å) were collected and merged. Data collection was by the step-scan method, with 0.3° intervals in ϕ and exposure times ranging from 60 to 77 minutes per frame. The net time required to collect the total of 717 data frames was 35 days. The data were processed with the programs DENZO and SCALEPACK.[13]

2.3 Refinement

Refinement of the structure was carried out in the resolution range 5-1.5 Å with 2.0 σ_F-cutoff, using the program X-PLOR[14] in which the topology and parameter files were modified for neutron crystallographic refinement by adding the necessary parameters for hydrogen and deuterium atoms. A randomly-selected set of data, representing 10 % of the reflections in the data in the 10-1.5 Å resolution range, was excluded from the refinement and was set aside for the calculation of R_{free}. Model building was performed using the program TURBO-FRODO.[15] Between each cycle of the refinement, $(2F_O - F_C)$ and $(F_O - F_C)$ maps were calculated in the resolution range 10 to 1.5 Å. Except for hydrogen atoms and water molecules, the atomic positions obtained from the previous X-ray study (PDB code 1brf) were used to phase the neutron data, and also used as the starting structure of the refinement. While all hydrogen atoms at non-exchangeable positions (mainly those of C-H bonds) were generated by the program X-PLOR, the potentially exchangeable hydrogen atoms which are bonded to oxygen and nitrogen atoms, were identified using the Fourier maps one by one in a stepwise procedure. H and D atoms could be identified as negative and positive peaks in the map, respectively. hydration water molecules were also identified individually using the Fourier maps. There were two kinds of shapes associated with the contours of water molecules, a sphere (or an ellipsoid) and a triangle. In the former case only an oxygen atom would be assigned to that position, and in the latter case a D_2O molecule would be placed at that position and oriented according to the shape. During each cycle of the refinement the positions of non-hydrogen/deuterium atoms and hydration water molecules were fixed. After the refinement the water molecules were located or oriented again.

§3. Results and Discussion

3.1 Statistics of data collection and refinement

With the present experimental arrangement, data could be recorded from the rubredoxin crystal to a resolution of 1.5 Å, and the completeness of the data set is currently at 76.8 %. The crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 34.32$, $b = 35.31$, $c = 44.23$ Å. Detailed statistics of data-collection and reduction are listed in Table I. The values of R and R_{free} were 36.8 % and 35.3 %, respectively, after the first cycle of refinement and 24.0 % and 26.3 % after the last one. A total of 301 hydrogen atoms and 40 deuterium atoms were included in the refinement of the structure, and 29 hydration water molecules have been identified thus far. Refinement statistics are also given in Table I.

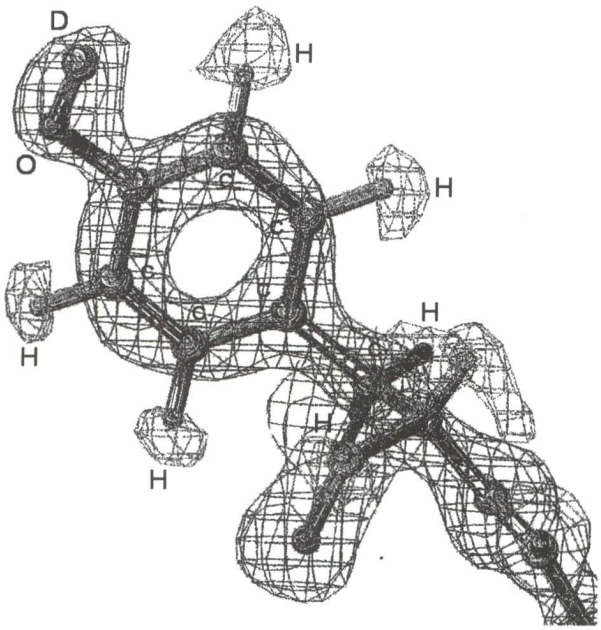

Fig. 1. $(2F_O - F_C)$ map around the residue Tyr10 and contoured at 2σ (gray) and -2σ (light gray) levels. Note that the D atoms (gray contours) can be easily distinguished from H atoms (light-gray contours), and that the direction of the O-D bond is unambiguously established. Also note the zero density region at the center of the aromatic ring.

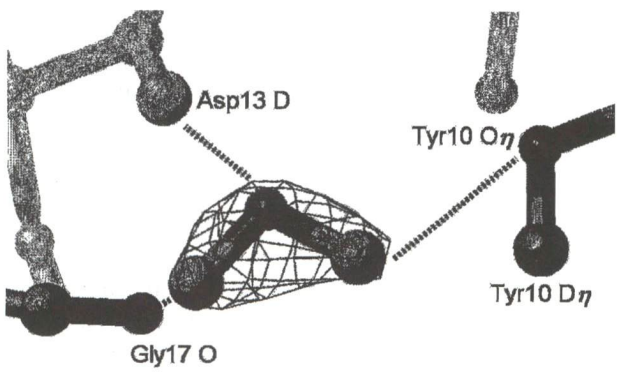

Fig. 2. A water molecule H-bonded to residues Tyr10, Asp13, and Gly17 and contoured at 3.5σ (gray) levels.

3.2 Hydrogen and deuterium atoms

The structure is still being analyzed and a full description will be presented in a future publication.[16] For this manuscript, a few representative structural features will be highlighted and discussed.

Figure 1 is an example of a $(2F_O - F_C)$ map, showing residue Tyr10 and the region around it. The scattering densities of H and D atoms are clearly visible in this figure. The atom bonded to oxygen could easily be identified as a D atom (instead of H) because it has positive scattering density (gray contour lines) in the map. On the other hand, the atoms bonded to carbon could be identified as hydrogen by virtue of their negative scattering densities (light-gray contour lines). A tyrosine residue has two possible O-D bond orientations. While X-ray diffraction usually cannot determine this orientation, it can be established with neutron crystallography as seen in Fig. 1. This is a clear example of how neutron diffraction can be useful in determining the detailed hydrogen-bonding structure. In addition, since the resolution of the present data set is 1.5 Å, the density for a phenol ring shows a clear hole at its center, a feature usually not evident in 2.0 Å-resolution maps.

Currently a total of 34 H/D atoms at potentially exchangeable sites do not have significant positive (deuterium) peaks at the expected positions. Of those, 17 are bonded to main chain nitrogen atoms, implying that those positions are not fully accessible to the H/D exchange process. The occupancy of H and D atoms will be discussed elsewhere.[16,17]

3.3 Hydration water molecules

At the present stage of structural analysis, a total of 29 hydration water molecules were found in the rubredoxin structure. Of those, 7 could be oriented unambiguously because the densities for those water molecules have a triangular shape. Figure 2 is a $(F_O - F_C)$ map (calculated without inputting any hydration water molecules) located near the three residues Tyr10, Asp13, and Gly17. According to the shape of the contours, the orientation of the D atoms of this water molecule could be determined. In this case it was found that the Dη atom of Tyr10 is not oriented towards this hydration water molecule, and so it is concluded that Tyr10 is serving as a H-bond acceptor, instead of a H-bond donor, to this particular water molecule. This is another example where, in certain favorable cases, neutron diffraction can reveal specific details of H-bonding patterns which would be difficult to obtain from X-ray data.

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