Proc. Int. Symposium on Advances in Neutron Scattering Research, Tokai, 2000
J. Phys. Soc. Jpn. **70** (2001) Suppl. A pp. 403–405

Neutron Crystallography of Hen Egg-White Lysozyme at pH4.9

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In order to elucidate protein stability and function, it is important to know states of protonation of each amino acid in the protein under different pH. To answer this problem, we have been studying the neutron protein crystallography of hen egg-white (HEW) lysozyme under different pH. Neutron diffraction experiments of single crystals of HEW lysozyme at pH4.9 were carried out with BIX-II at JAERI. The state of protonation of active site in the HEW lysozyme was investigated; there was a hydrogen (deuterium) atom bound to carboxylate oxygen atom of Glu 35 and no hydrogen (deuterium) atom bound to that of Asp 52. The results agreed with the proposed catalytic mechanism of active site in the HEW lysozyme.

KEYWORDS: neutron crystallography, neutron imaging plate, BIX-II, lysozyme, protonation

§1. Introduction

Neutron crystallography has become a powerful method for locating position of hydrogen (deuterium) atoms and bound waters of proteins.[1–4] Hydrogen atoms and bound waters play important roles in processes of biological activities of proteins as well as in stabilizing native structure of proteins. Catalytic activity of a protein, for example, has its optimized pH, indicating that protonation state of the active sites in the protein is essential in the process of catalytic activity. Moreover, charge distribution of the protein, which is dependent on pH of the solution, could affect thermodynamic behaviors of the protein. It is therefore important to investigate in detail the protonation state of each amino acid residue in the protein at various pH. Based on these motivations, we started a project which reveals the protonation of amino acid residues in the protein at various pH. We chose hen egg-white (HEW) lysozyme as a model protein. As a first step, we have started neutron diffraction measurements of single crystals of HEW lysozyme at pH 4.9.

Lysozyme, which cleaves oligosaccharides, has its optimum catalitic activity at pH 5.0. It is particularly important to know the protonation states of the active sites of the protein, glutamic(Glu) 35 and aspartic(Asp) 52, in understanding the mechanism of enzyme reaction of lysozyme. The recent study of neutron crystal structure analysis of HEW lysozyme at pH 7.0 showed that there were no observed hydrogen atoms bound to carboxylate oxygen atoms of glutamic(Glu) 35 and aspartic(Asp) 52.[1] We thus focus attentions on the protonation of the residues Glu 35 and Asp 52 in comparison with the results of HEW lysozyme at pH 7.0.

§2. Experimental

HEW lysozyme (6 times crystallized) was purchased from Seikagaku-kogyo co., and used without further purification. HEW lysozyme was crystallized using a concentration gradient method in D_2O at pH4.9,[5] in which $NiCl_2$ was used as a precipitant and pH of the protein solution was adjusted by adding small amount of concentrated HCl. Tetragonal crystal of HEW lysozyme was grown to the size of about 2 x 2 x 1 mm^3. Space group of the crystals was $P4_32_12$. Neutron diffraction experiments were carried out with the neutron diffractometer, BIX-II,[6] installed at the beam-port T2-3 in the guide hall of the reactor JRR-3M at JAERI. Monochromatized neutrons of a wavelength of 2.15 Å were used as an incident beam. BIX-II employs a neutron imaging plate as a detector,[7] the size of which is 400 x 520 mm^2 and the pixel size of which is 0.2 x 0.2 mm^2. Diffraction data were recorded with this neutron imaging plate at the crystal-to-detector distance of 150 mm. The oscillation-mode measurement of 0.4 ° intervals was employed. Exposure time of one frame was 11 hours.

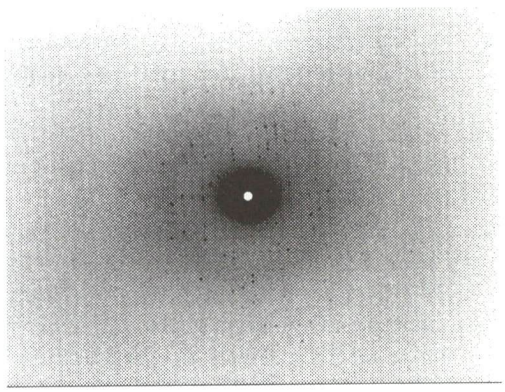

Fig. 1. An example of the neutron diffraction pattern of obtained by BIX-II

TableI. 2.0 Å resolution data-collection statistics.

Resolution(Å)	I/σ	R_{merge}†	R_{sym}‡	Completness(%)	No. of unique reflections
40.00 - 4.31	16.50	0.069	0.071	85.2	803
4.31 - 3.42	13.99	0.094	0.094	78.1	673
3.42 - 2.99	10.36	0.127	0.128	75.4	639
2.99 - 2.71	6.95	0.162	0.175	70.6	586
2.71 - 2.52	5.78	0.208	0.205	68.5	573
2.52 - 2.37	4.96	0.274	0.266	62.9	516
2.37 - 2.25	4.65	0.286	0.281	62.9	516
2.25 - 2.15	4.36	0.292	0.277	56.9	473
2.15 - 2.07	3.97	0.304	0.293	56.0	446
2.07 - 2.00	3.90	0.312	0.289	52.8	438
All hkl	8.50	0.110	0.140	67.3	5663

†R_{merge} =Σ ((I - <I>)^2)/Σ(I^2)
‡R_{sym} = Σ (| I - <I>|) / Σ I

§3. Results and Discussion

3.1 Data collection

Figure 1 shows an example of the neutron diffraction pattern obtained by BIX-II. Each image has 1300-1800 reflections. Total number of 303 frames was recorded in the measurements. The diffraction data were processed and scaled using the programs *DENZO* and *SCALEPACK*.[8] The refined unit-cell parameters were $a = b = 78.65$, $c = 37.35$ Å. The data-reduction statistics are presented in Table I. The number of unique reflections (more than 1σ) to 2.0 Å resolution was 5382, and the completeness was 63.96 %.

3.2 Refinement and model rebuilding

The structure refinement was carried out using *X-PLOR*.[9] The topology and the parameter files were modified for neutron crystallography so that hydrogen or deuterium atoms were included in the files.[1] HEW lysozyme structure (Protein Data bank entry code: 193L) at 1.33 Å resolution(pH 4.3) was used as an initial model. The cell constant was $a = b = 78.54$ and $c = 37.77$ Å. There are 967 hydrogen atoms in the lysozyme. We assumed that hydrogen atoms bound to carbon atoms were not replaced with deuterium. Hydrogen (deuterium) atoms bound to nitrogen or oxygen were removed from the initial model and all water molecules in 193L were not included in the initial model. 278 hydrogen (deuterium) atoms were deleted.

During the refinement process, 5083 reflections data between 7.0 and 2.0 resolution with $F_{obs} > 2.0\sigma$ (F_{obs}) were used, and 8 % of the data were set aside in order to calculate R_{free}.[10] In the initial step, the rigid-body refinement (40-cycles), followed by the positional refinement (80-cycles) were carried out. This was followed by simulated-annealing refinement with slow cooling, during which the temperature was decreased from 600 to 300 K. At this point of the refinement, the R-factor and the R_{free} for all the intensities in the 7.0-2.0 Å resolution range were 0.375 and 0.354, respectively.

The model building was carried out using *TURBO-FRODO* (Bio-Graphics). The $2F_o - F_c$ and $F_o - F_c$ scattering length density maps were calculated using 10.0-2.0 Å data.

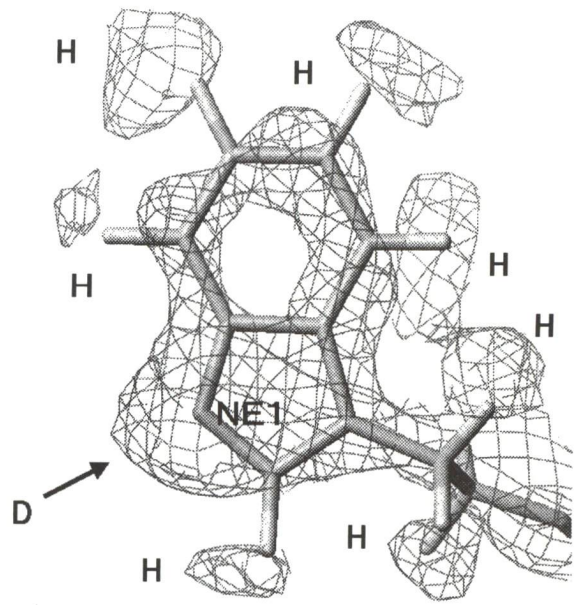

Fig.2. The $2F_o - F_c$ map around the indol ring of Trp111

In the refinement step, we checked the position of deuterium atoms of each of 129 amino resides one by one using $2F_o - F_c$ (1.5σ) and $F_o - F_c$ (2.0σ) maps. If appearance of a peak was consistent in both maps, the peak was identified as deuterium atom.

Figure 2 shows the $2F_o - F_c$ map around the indol ring of Trp111. This $2F_o - F_c$ map gives one example how hydrogen and deuterium atoms are identified. The negative density regions, denoted by the label H in Fig. 2, are identified as hydrogen atoms bound to carbon atoms because of the negative scattering length of hydro-

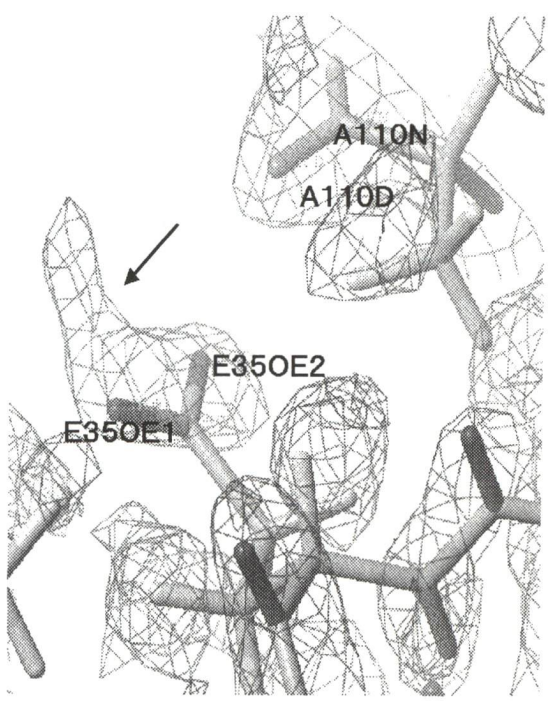

Fig.3. The $2F_o - F_c$ map around the carboxylate group of Glu 35

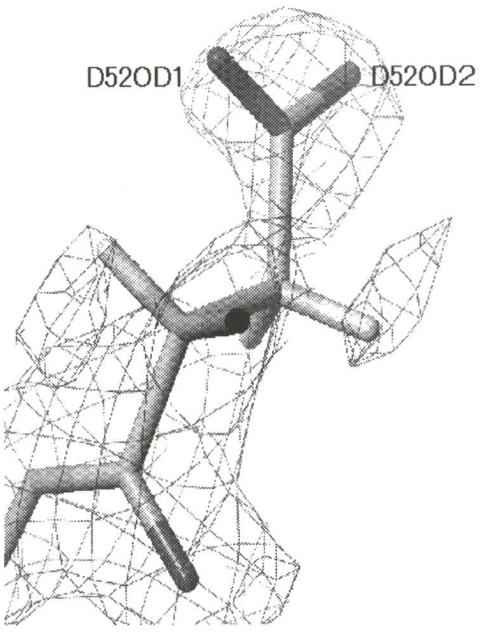

Fig. 4. The $2F_o - F_c$ map around the carboxylate group of Asp 52

gen. In contrast, as denoted by an arrow in Fig. 2, the positive scattering density appeared at the position of the hydrogen atoms bound to the nitrogen atom (NE1). This indicates that the hydrogen atom is replaced by the deuterium atom.

Water molecules were added to the model if the $F_o - F_c$ difference density had values more than 2.5σ level above the mean and the $2F_o - F_c$ showed density at more than 1.5σ level, and if each water molecule had reasonable distance from amino residues or other water molecules. After deuterium atoms and/or water molecules were added to the model, the positional refinement (80-cycles) and B-factor refinement(25-cycles) were carried out. 31 deuterium atoms and 30 water molecules were found in the refinement and model building so far. At this point, the R-factor and the R_{free} decreased to 0.289 and 0.328, respectively. The r.m.s. deviation from ideal geometry of bond lengths and bond angles were 0.004 Å and 1.272 °, respectively.

3.3 Catalytic site of lysozyme

In the proposed mechanism of reaction of the lysozyme with oligosaccharides,[11] the side chain of Glu 35 is protonated, whereas the side chain of Asp 52 is ionized at pH5.

Figure 3 showed the $2F_o - F_c$ map around the carboxylate group of Glu 35. As indicated by an arrow, the density continued from the position of the carboxylate oxygen atom labeled E35OE1 was observed, suggesting that this carboxylate oxygen atom is protonated.

On the other hand, the distance between carboxylate oxygen atom labeled E35OE2 and deuterium atom bound to nitrogen of Ala 110 was 2.31 Å, which indicates that hydrogen bond existed between them. In the case of the tetragonal HEW lysozyme at pH 7.0,[1] the hydrogen bond was formed between one water molecule and carboxylate oxygen atom labeled E35OE2.

Figure 4 showed the $2F_o - F_c$ map around the carboxylate group of Asp 52. It is clear that no hydrogen (deuterium) atoms bound to the carboxylate oxygen atom labeled D52OD1 and D52OD2. The results of protonation and de-protonation of catalytic site residues agreed with the proposed hydrolysis mechanism of the oligosaccharide by the lysozyme at pH5.

The information of the protonation state of each amino acid residue in the protein at various pH will provide us further data for the thermodynamic characterization of the proteins.

Acknowledgements

The authors thank Drs. A. Ostermann and K. Kurihara for helpful discussions. This work is supported in part by the Organized Research Combination System (ORCS) from Science and Technology Agency.

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