

VIII- II -1. Project Research

Project 5

PR5 Direct Observation of the Proton or Protonation in a Protein Molecule by Neutron and High Resolution X-ray Joint Analysis

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OBJECTIVES: Protein sciences are accelerated with use of a neutron and an X-ray beam to clarify the tertiary and quaternary structural information. Proton and/or protonation of the amino acids in the protein molecule affect a chemical reaction in the biological molecule, neutron is well useful to isolate findings of such reaction mechanisms and can determine a precise position and roles of hydrogen or protonation in active amino acids. Drugs, as reagents, anti-cancer compounds, play important roles in a host-protein molecule with electrons or protons, hydrogen (deuterium) atoms should be determined clearly to design a compound molecule. X-ray analysis is good method to find a whole molecule and neutron is sensitive and selective to hydrogen (deuterium), the both complementary usage or joint refinement of the protein molecule is very powerful technique to analyze a precise reaction mechanism with atomic resolution. Therefore a plan of this study, which is based on the last project (Hydrogen/Deuterium Exchanges in the Protein molecule), has been started in our institute.

I have called for members to carry out the examination of this purpose.

- (1) Structural investigation of the yeast 20S Proteasome in the highly structural organization, Y.Morimoto
- (2) Crystallographic studies on DNA affected by hydration and metalation. T.Chatake
- (3) Ligninase purification for a low carbon energy system by plant-type organic resources. Y.Hidaka
- (4) Deuterium culture of bacterial cells and purification of a deuterated kinase. Y.Yanagisawa

There are the following research activities in this year.

RESULTS:

25P5-1

Y. Morimoto, T. Murakami, Y. Morita, M. Unno, I. Hisatome and H. Yamaguchi reported "Weak interaction of an inhibitor in the 20S proteasome elucidated by a crystal structure analysis". A newly inhibitor of the 20S proteasome and its complex is described. The compound binds to the S1-pocket inside the whole 20S particle and packed among two or three β -strand of the 20S proteasome, and interacts with some amino acids of this 20S proteasome. And the other compounds have been determined and checked with 20S

whole structure complexes. Common structural characters are found in such compounds whose aromatic ring interacting a surrounding amino acid residues in the 20S proteasome subunits. They are good points in a drug designing of a drug compounds. Furthermore, the structural analysis for some genetic mutants covered an active amino-acid by changing to be hydrophobic residues in a yeast are now under going.

25P5-2

T. Chatake, S. Fujiwara, Y. Morimoto reported "Crystallographic study of hydration and metal interactions of DNA" of the result for the newly time of flight diffractometer in the JPARC. The neutron analysis demonstrated hydrogen and metal interaction of Z-DNA. (1) At the GpC steps, equilibrium between the Z_I and Z_{II} conformations was frequently observed. (2) At the CpG steps, the phosphate groups exhibited rotational fluctuation. (3) In the minor groove of Z-DNA, alternative positions of water molecules were found. These features suggested that divalent metal cations would contribute the stabilization of Z-DNA conformation.

25P5-3

Y. Hidaka, M. Fujiwara, T. Nakanishi, S. Shimamoto, and M. Miyazawa reported "Identification and characterization of proteolytic enzymes derived from *Nephila Clavata*" focusing to a newly proteolytic enzyme in a spider. Authors have caught spiders (Jorou-kumo) in our institute and obtained a new code of gene for such unknown enzyme. In his conclusion, spider protease was extracted from its digestive fluid and exhibited strong protease activity. The protease can be classified as a Ca^{2+} -dependent carboxypeptidase, based on the results of protease inhibition assays.

25P5-4

Y. Yanagisawa, M. Sakanushi, M. Ishiyama, S. Onuma, S. Fujiwara, T. Matsuo, T. Chatake, and Y. Morimoto reported "Preparation of nattokinase for neutron experiments". They established purification and crystallization protocols of nattokinase from deuterated solution, and characterized a menaquinone-7 (MK-7) producing vitamin K_2 in *Bacillus subtilis* natto by a dynamic light-scattering method. Results show a molecular weight of the MK-7 was 90 kDa, and assuming that MK-7 was the complex of biochemical molecules and vitamin K_2 . It is noteworthy that aggregates were observed at $\sim 1000 \text{ \AA}$, and its distribution differed depending on temperature.

Weak Interaction of an Inhibitor in the 20S Proteasome Elucidated by a Crystal Structure Analysis

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INTRODUCTION: Proteasomes are the multicatalytic protein complexes with huge molecular weight. It is well known that the ubiquitin proteasome system plays an important role in regulated proteolysis. Proteasomes are known to act as antigen-processing enzymes responsible for the generation of peptide ligands presented by major histocompatibility complex (MHC) class I molecules. The 20S proteasome forms barrel shape and consists of 4 protein rings arranged each other in parallel plain. Upper two rings are arranged symmetrically with lower two rings and they are called α - and β -rings from outer to inner side. Each of 1, 2 and 5 β subunits has different enzyme activities; 1 has caspase-, 2 tryptic- and 5 chymotryptic-like activities. It was found recently that inhibition of 20S proteasome activity resulted in decrease and disappearance of cancer cells. Therefore the inhibitor of 20S proteasome serves as a new anticancer reagent. We found a new protease inhibitor compound-A, which interacts weakly against proteasome. In order to elucidate the binding mode of the compound-A to proteasome, structure determination of the 20S proteasome complexed with compound-A was carried out.

EXPERIMENTS: Yeast 20S proteasome tagged with affinity peptides was prepared in yeast. Cells were homogenized by glass beads and the crude extracts were purified by M2 affinity chromatography and Mono-Q anion exchange one. Isolated 20S proteasome was concentrated by ultrafiltration, and co-crystallized with small ligand molecules. Vapor diffusion method was applied. Crystals are isomorphous as described in the previous paper and belong to the space group $P2_1$. Diffraction images were recorded at 100 K by using ADSC Quantum 210r CCD detector at NW12A of Photon Factory, Tsukuba, Japan. Initial phases were determined by molecular replacement method using the structure of the yeast 20S proteasome as a starting model and the structure model

with a ligand was refined by using Refmac5 in CCP4 program package with an R value of 16.5% at 2.85Å resolution.

RESULTS: The electron densities have been observed at the active site as mentioned above in the β -ring. Binding site of compound-A is closed to Tyr170 and Thr1 of the $\beta 5$ subunit. The compound-A binds weakly to those residues by hydrogen bondings. It is quite different from the binding mode of the known potent proteasome inhibitor bortezomib. Until last year, latest proposal, some compounds have been found in the 20S proteasome complex and reported. In the results, the S1 pocket located inside 20S particle has a chymotryptic-like active site composed of Tyr(135), Gly(128) and VAL(129) and holds a compound-A (Fig.1). Such an aromatic ring interacts with those amino acid residues by ring-stacking force. It may confirm the other compound reagents bind on the same place or position, so another three compounds have been determined in the 20S complex analysis by synchrotron radiation sources. Results show structurally similar compounds present a same binding-mode by aromatic effects. Enzyme activities, however, are different with each other, compounds (-B,-C and -D) have no effective assay by dose-dependent manner (data not shown).

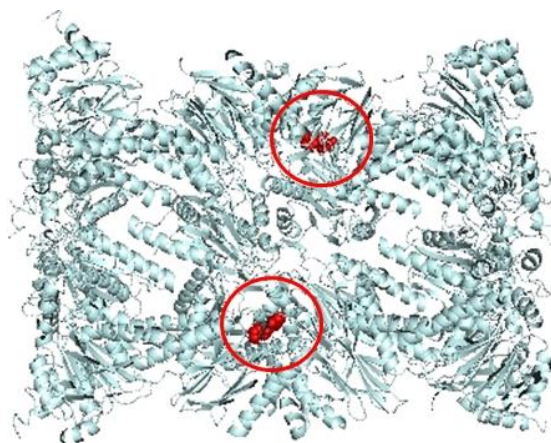


Fig.1 Structural evidences for compound-A binding to the 20S proteasome. Overall structure of the 20S proteasome in complex with compound-A. Compound-A is colored by red for its carbon atoms. The NCS (non-crystallographic symmetry)-related subunits equally bind compound-A.

The tumor suppressor p53 playing a pivotal role and p53-FLAG expressed in COS7 cells are controlled by the 20S proteasome with a concomitant accumulation of its ubiquitinated form. In order to confirm the degradation of those complexes, co-expression of the C438A mutant of Mdm2 with p53-FLAG is now under progress.

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INTRODUCTION: Structural polymorphism of DNA is an important clue used to understand the varieties of structures and functions of DNA molecules. In particular, humidity and metal cations have been thought to affect the structural polymorphism. Our aim in this project is to study the effects of hydration and metal-interaction on DNA duplexes using the combination of X-ray and neutron crystallographic techniques. In the previous report, we reported the preliminary neutron TOF experiment of Z-DNA at J-PARC [1]. This experiment was carried out using non-deuterated crystal of Z-DNA d(CGCGCG). Because neutron diffraction data of deuterated crystal of the Z-DNA was collected [2]. The combination of two diffraction data of D₂O and H₂O crystals is expected to provide D/H contrast neutron maps of solvent regions. In this FY2013, further neutron experiment was cancelled, and was re-scheduled at FY2014, accordingly structural study of DNA hydration was postponed. On the other hand, X-ray crystallographic analyses of interaction between Z-DNA and metal cations were accomplished [3,4]. In this report, Z-DNA structures in presence and absence of divalent metal cations (Mg²⁺ and Ca²⁺) are written.

EXPERIMENTS: DNA hexamer d(CGCGCG) was crystallized in the temperature controlling method reported previously [5]. 4 μ L droplets of 2 mM Z-DNA, 30% 2-methyl-2,4-pentanediol, 20 mM sodium cacodylate (adjusted at pH7.0), and different metal salts (summarized in Table 1) were sealed under paraffin oil, and were cooled from 343 K to 293 K at a rate of 5K/day. Crystals were flash-cooled using 100 K nitrogen gas, and X-ray diffraction data sets were collected at beamline 38B1 in SPring-8. Initial phase of diffractions were determined by the molecular replacement method, and then model building and refinements of atomic parameters were carried out using Coot and Phenix, respectively.

Table 1. Statistics of X-ray experiments.

Name	High-Mg ²⁺	High-Ca ²⁺	No-M ²⁺
Metal ion	500 mM Mg ²⁺	500 mM Ca ²⁺	40 mM Na ⁺
SP	P3 ₂	P3 ₂ 21	P2 ₁ 2 ₁ 2 ₁
<i>d</i> _{min} (Å)	1.3	1.3	0.98
<i>R</i> _{merge}	0.054	0.072	0.04
<i>R</i> -factor	0.203	0.192	0.127
<i>R</i> _{free}	0.274	0.232	0.154

RESULTS: Three kinds of Z-DNA crystals were used for X-ray structural analyses. High-Mg²⁺, High-Ca²⁺, and No-M²⁺ were grown in solutions containing 500 mM

Mg²⁺, 500 mM Ca²⁺, and 40 mM Na⁺ as metal cation, respectively. All the three structures could be determined at high-resolution (0.98~1.3 Å). Interestingly, molecular contacts between neighboring DNA duplexes changed depending on species and concentrations of metal cations. In particular, the crystals at high concentration of divalent metal cations took novel space groups (P3₂ for High-Mg²⁺ and P3₂21 for High-Ca²⁺, respectively).

Striking features of Z-DNA observed at high concentrations of divalent metal cations were in the following [3],

(1) One Z-DNA duplex interacted with its six neighboring DNA duplexes through coordination bonds of P-O...(Mg²⁺ or Ca²⁺)...O-P.

(2) In order to take the coordination bonds, all phosphate groups of GpC steps took Z_{II} conformation, while Z_I conformation were usually observed in Z-DNA duplexes.

The two features are distinct from normal Z-DNA except Z'-DNA that was reported only once in the early period of nucleic acid crystallography [6]. This type of DNA-metal interaction and packing is expected to be an appropriate assembly model for Z-DNA duplexes at high concentrations of divalent metal cations.

On the other hand, in absence of any divalent metal cation, interaction between Z-DNA duplexes, which had been often observed in previous studies, was conserved. However, lack of divalent metal cation caused structural fluctuations of Z-DNA and its hydration [4].

(1) At the GpC steps, equilibrium between the Z_I and Z_{II} conformations was frequently observed.

(2) At the CpG steps, the phosphate groups exhibited rotational fluctuation.

(3) In the minor groove of Z-DNA, alternative positions of water molecules were found.

These features suggested that divalent metal cations would contribute the stabilization of Z-DNA conformation, and the structural fluctuations would be more or less happened in Z-DNA in organisms.

In near future, neutron structural analysis is expected to demonstrate relationship between these fluctuation and dynamic behavior of Z-DNA from the viewpoint of hydrogen an hydration structures of Z-DNA.

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PR5-3 Identification and Characterization of Proteolytic Enzymes Derived from *Nephila Clavata*

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INTRODUCTION: Spiders hunt insects using a web net. The fact that they eat them without chewing, indicate that spiders possess highly efficient digestive enzymes [1]. Our previous studies suggest that spider's proteolytic enzymes are able to digest synthetic spider dragline amyloid fibers [2,3]. Thus, the spider protease has the potential ability to digest amyloid fibers including pathogenic β -amyloid, such as amyloid fibrils, that are associated with the development of Alzheimer's disease [1]. Therefore, we purified, identified and characterized the enzymes derived from *Nephila Clavata*.

EXPERIMENTS: The spiders (*Nephila Clavata*) were collected in the field of Kyoto University Research Reactor Institute and the spider saliva including proteolytic enzymes was obtained from *Nephila Clavata* (50 heads) by electrical stimulation. The extracts were stored in a deep freezer until use.

Casein protease assay [4] of the crude spider enzymes was performed, as previously reported [1]. Peptide substrates for the assay of the protease activity of spider enzymes were chemically synthesized by the Boc solid phase method, treated with hydrogen fluoride, and purified by reversed phase HPLC [1, 4].

The N-terminal protein sequence was determined by the Edman degradation. First, the spider saliva was applied to SDS-PAGE, and the protein bands were electrically transferred to a PVDF membrane. The electro-blotting of spider proteases was performed using the semi-dry blotting method.

RESULTS: Spider's digestive fluid that includes proteolytic enzymes was obtained from *Nephila Clavata* by electrical stimulation using a micropipet. The extracts were applied to SDS-PAGE and the enzymatic activity of the protein bands was estimated by a casein protease assay [4]. Two protein bands showed protease activities and their molecular weights were estimated to be approximately 21.9 and 19.5 kDa, based on the SDS-PAGE analysis [1]. To characterize the enzymes, an inhibition

assay was performed using several types of inhibitors, including PMSF and EDTA in the Casein protease assay. PMSF weakly inhibited the Ser-protease activity and EDTA and EGTA completely inhibited the activity. This result indicates that the protease can be classified as a Ca^{2+} -dependent protease [1].

To further characterize the spider protease, FLGLPAKADA-NH₂ and SIFKTLRTIA-NH₂ were synthesized as model peptide substrates and treated with the crude spider protease. The reaction mixtures were separated and analyzed by reversed phase HPLC at each time point of the digestive reaction and the eluted fractions were analyzed by MALDI-TOF/MS. The results revealed that the protease was able to digest the synthetic peptide from the C-terminal residues, regardless of whether the C-terminal group was a carbamide moiety [1]. In addition, the results also indicated that the Arg and Lys residues are slightly resistant to protease digestion. Therefore, combined with the above findings, these results indicate that the spider protease can be classified as a Ca^{2+} -dependent carboxypeptidase although the inhibitors were not able to completely suppress the protease activity of the enzyme under the conditions used in this study. Several types of spider's proteolytic enzymes have been reported and they are mainly classified as a trypsin-like metalloprotease, such as Astacin [5]. However, our results suggest that the spider protease should be classified as a carboxypeptidase and possesses superior activity to other carboxypeptidases, providing a new enzymatic tool for biochemical study.

In conclusion, Spider protease was extracted from its digestive fluid and exhibited strong protease activity. The protease can be classified as a Ca^{2+} -dependent carboxypeptidase, based on the results of protease inhibition assays. The determination of the protein sequence is currently underway.

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PR5-4 Preparation of Nattokinase and Menaquinone-7 for Neutron Experiments

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INTRODUCTION: Natto is a Japanese traditional food, and produces several chemical compounds, which contribute for health. Structural studies of two biochemical compounds are in progress for revealing their molecular mechanisms.

Nattokinase (NK) is a fibrinolytic enzyme, which is produced in abundance when soybeans are fermented to natto by *Bacillus subtilis natto* [1]. In our previous study of this project, the non-hydrogen structure of NK had been determined by X-ray crystallography [2]. However, its substrate specificity, which is apparently different from the other fibrinolytic enzymes could not be explained completely only by the X-ray result. In order to fully understand the molecular mechanism of NK, we are trying neutron crystallographic study, which will provide the 3D structure including hydrogen atoms. Protein deuteration is usually an essential process in neutron study, because deuterium has larger coherent neutron scattering and smaller incoherent scattering than those of hydrogen. Previously, we succeeded in culturing *Bacillus subtilis natto* in deuterated medium, and reported [3]. Additional experimental results are written in this report.

Menaquinone-7 (MK-7) is vitamin K₂ produced in *Bacillus subtilis natto*. Interestingly, MK-7 has hydrophilic property, while vitamin K₂ has hydrophobic property alone. Therefore, MK-7 is thought of a water-soluble biochemical complex, and it is expected as a dietary supplement. However, the 3D structure of MK-7 is not revealed yet. Here, the procedure of production of high purity MK-7 for analytical experiments and the first solution scattering data of MK-7 is reported.

EXPERIMENTS:

Nattokinase (NK): Successive cultivations of *Bacillus Subtilis Natto Miyagino* in deuterated medium was carried out in the manner described in our previous report [4]. Tendency of deuteration of culture medium was estimated by measuring neutron transmission at 4CND at KUR. The first purification was carried out at a laboratory of Chiba Institute of Sciences (CIS), and the second and later purification and analytical experiments were carried out in Tracer laboratory of Research Reactor Institute of Kyoto University (RRI).

Menaquinone-7 (MK-7): All cultivation of *Bacillus Subtilis Natto Miyagino* was carried out in not deuter-

ated but undertreated medium. After 7-days or 14-days cultivation, the cultivated medium was filtered and concentrated at the CIS laboratory, and purification was carried out at TL in RRI. After the final purification, the size of particle diameter of MK-7 was analyzed by the dynamic light scattering (DLS) method.

RESULTS:

Nattokinase (NK): In the successive cultivation of *Bacillus subtilis natto* was carried, where D₂O concentration of culture medium was increased in steps of 25%. The D₂O concentration reached 100% in eight successive cultivations, while considerable numbers of trials were end in failure (NK activity lost). Deuteration of the cultured medium was confirmed by neutron experiments. pH of medium suggested that the D₂O-resist strain resistant would lost the pH regulating system. In order to perform large-scale cultivation, the control of the pH of the medium would be necessary.

Menaquinone-7 (MK-7): Purification was carried out by the combination of four times of DEAE sepharose ion exchange chromatography and sephacryl S-200 gel filtration chromatography. Molecular weight of the purified MK-7 was assumed to be ~90 kDa by assessing the chart of the gel filtration chromatography. Fig. 1 shows the size distribution by DLS of 0.5 mg/mL MK-7. The diameter of the particle in the MK-7 solution was calculated to be ~40 Å, and this value would reasonable, assuming that MK-7 was the complex of biochemical molecules and vitamin K₂, of which weight was 90 kDa. It is noteworthy that aggregates were observed at ~1000 Å in this chart, and its distribution differed depending on temperature.

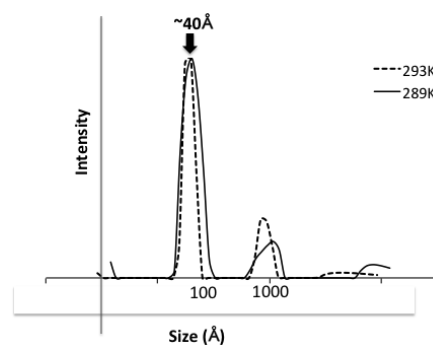


Fig. 1. Dynamic light scattering of MK-7.

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