VIII-II-1. Project Research

Project 9

PR9 Deuterium Exchages in the Biological Macromolecules for a Neutron 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 characteristics. Especially the neutron is very sensitive and selective to deuterium rather than a hydrogen, deuterium labeled molecules are available for the structural investigation in a complex and aggregation of the protein molecule. We should exchange hydrogens to deuterium atoms in the molecule in order to detect well the labeled molecule, therefore a plan of the deuterium exchange method suitable for a neutron usage in our institute has been started.

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

(1) Structural investigation of the 20S Proteasome and Anesthetic agents, Y.Morimoto
(2) Neutron Scattering Studies on Proteasome Component, M.Sugiyama
(3) Characterization of the human crystalline, N. Fujii
(4) Technical development for a large-scale jar-fermentor, T. Saito
(5) Development of deuterium carbon sources, S. Fujiwara
(6) Deuterium DNA and Protein purification, T. Chatake

There are the following research activities in this year.

RESULTS:

21P9-7 and 21P9-5

Y. Morimoto, U. Bahudin, K. Nishio, T. Saito and I. Hisatome are reported “Structural investigation of the 20S Proteasome and Anesthetic agents” of the result “The result leads us to more crystallization with various concentration of the drugs. In contrast, deuterated drugs could detect its positional coordinates in the 20S particle by a neutron scattering method.“

21P-1

M. Sugiyama and Y. Morimoto are reported “Neutron Scattering Studies on Proteasome Component” of the result “A deuterated sample could clearly show an appreciable model by use of neutron scattering. Biological implication for a proteasome activity are affected by such a structural exchange in the whole particle”.

21P9-2

T. Ishikawa, T. Chatake, A. Kawaguchi, Y. Yanagisawa, S. Fujiwara, and Y. Morimoto are reported “Study of Effect of Deuteration on Crystal Structure of Proteinase K by High Resolution X-ray Diffraction Technique” of the result “Atomic coordinates of PK-H and PK-D were precisely determined in the present analyses. All main-chain and almost side-chain could be perfectly determined. Some side-chains had alternative conformations in same fashion as reported earlier . In the earlier report, crystal was obtained using $NaNO_3$, therefore some NO_3^- anions were included in the crystal structure as impurity, and some side-chain were distorted. On the other hand, the present crystals were obtained from PK, buffer, $Ca(Ac)_2$ and PEG8000. As the result, the distortions due to NO_3^- were modified and, hence these crystal structures could be thought of natural form”.

21P9-3

Y. Yanagisawa, T. Chatake[1], T. Ishikawa[1], Y. Morimoto[1], I. Yasuda, T. Ohsugi[2], and H. Sumi are reported “Purification and Crystallization of Nattokinase from *Bacillus subtilis natto”* of the results “There were a few crystals in the solution and their shapes seemed to be better than the plate-like NK-HIC crystals, which were used in the preliminary X-ray experiment. The optimization of crystallization condition is in progress”.

21P9-6

T. Chatake, S. Fujiwara, and Y. Morimoto are reported “Crystallization of Ribonuclease A and H/D Exchange for Neutron Diffraction Experiment” of the result “Neutron diffraction experiment of the RNase-H_2O was carried out using the BIX-3 diffractometer at the JRR-3M reactor in Japan Atomic Energy Agency. Neutron diffraction data enough to determine the three dimensional structure of this crystal could be collected in two weeks. Neutron diffraction data collection of the RNase-D_2O is scheduled in the first half of FY2010”.

21P9-4

N. Fujii has prepared a construct of the human crystalline and mass spectroscopic studies in the year, and the results are now in progress.

PR9-1 Neutron Scattering Studies on Proteasome Component

M. Sugiyama and Y. Morimoto

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INTRODUCTION: Mammals have defensive systems in their safety living. One of typical examples is a proteasome-ubiquitin system. In such a system, there are two main protein complexes: 20S proteasome, which is known as a degrading factory for intracellular proteins, and its activator protein. Invasion of foreign substances is a trigger to produce Proteasome Activator 28 (PA28) induced by an interferon γ, and then the 20S proteasome makes a complex with two PA28s. The target protein is specified by PA28 and degraded by the 20S proteasome. According to this, the 20S proteasome and PA28 complex regulates the processing of antigenic proteins for presentation by the MHC class I pathway. However, there is another activator protein, PA700 for the 20S proteasome. PA700 also connects to the 20S proteasome and then the complex has another function; the complex breaks a ubiquitinated protein up. Therefore, it is very important to elucidate the process of the connection to the 20S proteasome of PA28 in order to understand its regulation and/or connecting mechanism. In addition, it has been also reported that a homo-oligomer PA28α is incapable of activating the 20S proteasome. In view of the situation, we have made an attempt to reveal the packing structure of the subunits in hetero-oligomer of PA28 by utilizing a contrast variation method of SANS.

EXPERIMENTS: Sample preparation and various-sample condition (concentration, pH and so on) are carried out at the KUR D-shop Lab using a yeast expression system. The observed SANS data were corrected for background, cell, buffer scatterings and the transmissions.

RESULTS: In order to examine the experimental result, the SANS profile and the gyration radius of PA28 should be simulated according to its atomic coordinates. For this simulation, we should know the atomic coordinates of PA28 in a heavy water solution. At first, the atomic coordinates of PA28*α* which was solved by X-ray diffraction were obtained from Protein Data Bank (PDB): PDB ID is 1AVO[1]. However, there is no atomic coordinate of hydrogen in this coordinates set even though neutron scattering cannot ignore hydrogen. Therefore, the hydrogen atoms were added at the most probable position to the data by using program CNS[2]. In addition, the all replaceable hydrogen atoms were exchanged with deuterium atoms using CNS with a neutron-dedicated parameter file because the solvent of sample solution was heavy water.

At first, SANS experiment was performed with undeuterated hetero-oligomer of PA28. The figure 1 shows experimental and calculated SANS profiles: the calculation was performed using PDB data of PA28α. The calculated SANS profile of the dimer of PA28 well-reproduces the experimental one.

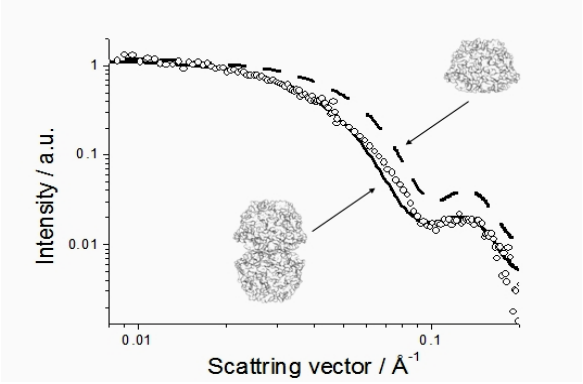

Fig.1. Scattering Intensities on the monomer and dimer particles.

Next, in order to clarify the packing structure of α and β subunits in the hetero-oligomer, the SANS experiment was performed with partially deuterated PA28: the hetero-oligomer consists of deuterated α subunits and hydrogenated β ones. Figure shows experimental and calculated SANS profiles of two probable models at matching point of β subunits. The result indicates (Fig.2) that an alternative packing model is preferable one.

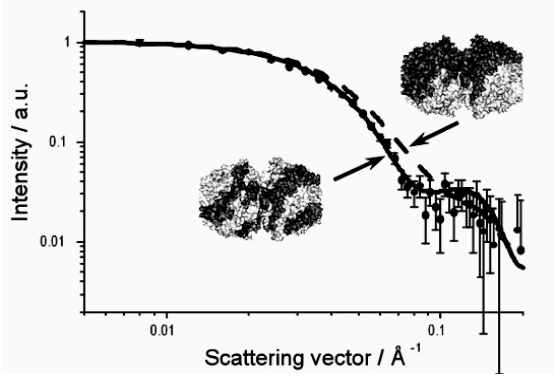

Fig.2. Scattering Intensities on the alternative packing models.

A deuterated sample could clearly show an appreciable model by use of neutron scattering. Biological implication for a proteasome activity is affected by such a structural exchange in the whole particle.

REFERENCES:

[1]Knowlton *et al*. (1997)

[2]Brünger. (1998)

採択課題番号 21P9-1　蛋白質複合・凝集体のX線・中性子溶液散乱法による動態解析　プロジェクト（京大・原子炉）杉山正明、森本幸生

PR9-2 Study of Effect of Deuteration on Crystal Structure of Proteinase K by High Resolution X-ray Diffraction Technique

T. Ishikawa, T. Chatake, A. Kawaguchi, Y. Yanagisawa[1], S. Fujiwara[2] and Y. Morimoto

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INTRODUCTION: Deuteration of macromolecules is a popular technique for neutron diffraction study. An incoherent neutron scattering from hydrogen atom is much longer than those of non-hydrogen atoms, therefore biomolecular samples are pre-deuterated by heavy water (D_2O) before neutron diffraction experiment. D_2O is a solvent molecule, of which chemical and physical properties resemble to natural water (H_2O). Therefore, D_2O sometimes is used as a substitute for H_2O. In studies of structural biology, D_2O is used for 1H-NMR spectroscopy and neutron crystallographic analysis. In case of neutron crystallographic analysis, D_2O is the essential solvents for crystallization

It is an important subject to investigate the influence on bio-macromolecules. So far, some structural studies have been reported, however it is not clear. In our study, the evaluation of the effect of D_2O on crystallization of proteinase K (PK) [1] was carried out by high resolution X-ray crystallographic analysis. In the previous study, we succeeded in obtaining good crystals. High-resolution X-ray analysis showed the degradation of crystals by deuteration. Nevertheless it would be negligible [2]. In the present study, we refined D_2O- and H_2O- crystal structures of PK, in order to investigate structural differences by deuteration.

EXPERIMENTS: PK was purchased from Merck Co. Purification and crystallization were carried out at TL building in RRI as described earlier [2]. X-ray data collection was carried out at Photon Factory. 1.1Å X-ray data sets of PK crystals of 100% D_2O and 100% H_2O (PK-H and PK-D) were used for structural determination. Refinement and model-building were performed with the program Refmac5 and Coot [3]. Data statistics after refinements were summarized in Table 1.

Table 1. Statistics of structure determination

Crystal	PK-H	PK-D
Initial model	1IC6 in PDB	PK-H
No. of reflections	91,586	92,622
For free R	4,832	4,874
Effective resolution	10.0-1.1 Å	10.0-1.1 Å
R_{work}	11.6	12.9
R_{free}	14.7	15.9

RESULTS: Atomic coordinates of PK-H and PK-D were precisely determined in the present analyses. All main-chain and almost side-chain could be perfectly determined. Some side-chains had alternative conformations in same fashion as reported earlier [4]. In the earlier report, crystal was obtained using $NaNO_3$, therefore some NO_3^- anions were included in the crystal structure as impurity, and some side-chain were distorted. On the other hand, the present crystals were obtained from PK, buffer, $Ca(Ac)_2$ and PEG8000. As the result, the distortions due to NO_3^- were modified and, hence these crystal structures could be thought of natural form. Moreover, in these high-resolution analyses, a glycerol molecule used as a cryo-protectant were found in both of the crystal structures.

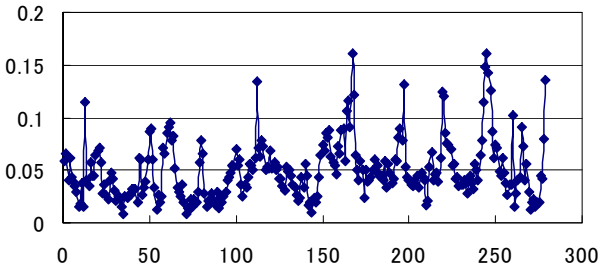

Fig. 1. Root-.mean-square differences of atoms of main-chain between PK-H and PK-D.

Figure 1 shows the root-mean-square differences of main-chain between PK-H and PK-D, indicating the small structural changes due to deuteration. Although some residues have relatively larger differences than averaged r.m.s.d, it is lower than 0.17 $Å^2$. In addition, these residue correspond to high B-factor regions of PK in the crystals. Hydration of PK-D and PK-H were also resemble each other. It can be thought that no striking structural changes occur in deuterated solution, in case of crystallization of PK using PEG8000 as precipitant.

It can be concluded that polyethylene glycol would be a good reagent for crystallographic study of deuterated proteins such as neutron diffraction study. The degradation of protein crystals and the changes of their crystal structure would be small and negligible.

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[1] P. J. Sweeney, J. M. Walker, Meth. Mil. Biol. **16** (1993) 305-311.
[2] T. Chatake *et al.*, KUR Progress Report 2007 (2008) 206.
[3] Collaborative Computational Project, Number 4. Acta Crystallogr. **D50** (1994) 760-763.
[4] Betzel *et al.*, Biochemistry **40** (2001) 3080-3088.

採択課題番号 21P9-2　　ＤＮＡの重水素化サンプルの作成と 4CND による検査　　プロジェクト
（京大・原子炉）茶竹俊行、石川卓哉、川口昭夫、森本幸生（原子力機構）藤原悟
（いわき明星大・薬）竹中章郎

PR9-3 Purification and Crystallization of Nattokinase from *Bacillus subtilis natto*

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[1]Research Reactor Institute, Kyoto University
2Department of Life Science, Kurashiki University of Science and the Arts

INTRODUCTION: Nattokinase (NK) is abundantly contained in Japanese traditional food *natto* (1). NK is a 275 a.a. protein (M.W.=27,724) , which is produced by *Bacillus subtilis natto*. We previously reported that it would be a kind of serine proteases and has thrombolytic activity (2-4). NK would be one of candidates for neutron protein crystallography, because a large amount of native NK can be easily obtained from *Bacillus subtilis natto* and a clinical application would be expected. In the present study, we purified NK and crystallized it for diffraction experiments. We succeeded in obtaining the first diffraction image of NK in a preliminary X-ray experiment.

EXPERIMENTS: Purification and crystallization were carried out at TL building in RRI. Two methods were used for purification of NK. They were hydrophobic interaction chromatography (HIC) and gel filtration chromatography, respectively. (1) In the HIC methods, NK was precipitated by 1.5 M ammonium sulfate (AS), and then the precipitation was dissolved in a large quantity of solution containing 25mM Tris-HCl (pH6.8), 2.0 M AS and 2mM phenylmethanesulfonyl fluoride (PMSF). The solution was purified by GE butyl-sepharose FF (1ml) column mounted in AKTA prime FPLC (GE healthcare corp.). The gradient method from 2 M AS to 0 M AS was performed with the flow rate of 1.0 ml/min and the total time of 100 min. NK was eluted at 1.5 – 1.75 M AS. It was desalted GE Sephadex G-75 (ϕ1.5x36 cm) and was concentrated up to 2-5 mg/ml. (2) In the GC methods, NK was deactivated in a solution containing 50mM Tris-HCl (pH7.5), 5mM calcium chloride and 2mM PMSF at 4 degree for 12 hours. After the deactivation, it was desalted by the first gel filtration using GE Sephadex G10 (ϕ2.0x15 cm), and then it was further purified by the second gel filtration GE Sephacryl S200 (ϕ1.5x40 cm). The final NK-GC solution contained 6.4mg/ml NK-GC, 50mM Tris-HCl (pH 7.5), 100 mM Sodium chloride and 5 mM $CaCl_2$.
NK purified by HIC (NK-HIC) was crystallized by Crystal Screen 1 (Hampton Research corp.). NK purified by gel-filtration chromatography (NK-GC) was crystallized by Crystal Screen 1 and Crystal Screen 2. In the both cases, crystals of NK could be obtained.
Crystals of NK-HIC was used for a preliminary X-ray diffraction experiment

RESULTS: Crystals of NK-HIC and NK-GC were obtained in the present study. Two types of NK-HIC crystals were observed. One of the two was a needle-like crystal. A lot of micro needle-like crystals were grown in the crystallization solution. 15% SDS electrophoresis showed that these crystals actually contained NK (Fig 1a). However, they were too small to get X-ray diffraction. Another was a plate-like crystal as shown in Fig. 1b. It was large enough for an X-ray diffraction experiment. It was soaked into 50% MPD solution about 5 sec and was flash-cooled by 100 K nitrogen gas before an X-ray experiment. As the result, the first X-ray diffraction images could be collected. The effective resolution was estimated to be 3.5 Angstrom. Data collection could not be succeeded due to ice rings.

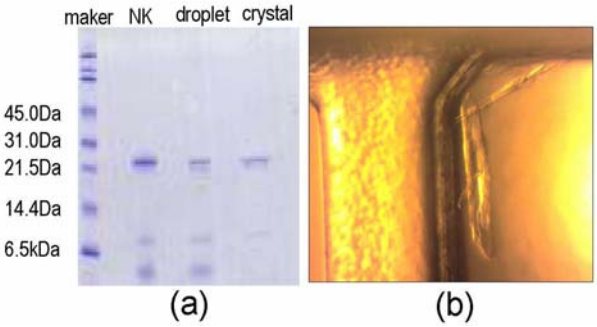

Fig. 1. Results of crystallization of NK. (a) 15% SDS electrophoresis. Needle-like NK crystals contain NK (M.W. 27.8kDa). (b) A photograph of plate-like NK crystals. Photographs of NK-HIC crystals.

Three kinds of crystals of NK-GC could be observed. The two of three were similar to the needle-like crystal and the plate-like crystals of NK-HIC, respectively. In case of NK-GC, prism-like crystals could be grown in one of crystallization solutions of Crystal Screen 2. There were a few crystals in the solution and their shapes seemed to be better than the plate-like NK-HIC crystals, which were used in the preliminary X-ray experiment. The optimization of crystallization condition is in progress.

REFERENCES:

[1] H. Sumi *et al., Experientia.,* **43**(1987) 1110-1111.
[2] H. Sumi *et al.*,Acta Haematol., 1990;**84**(3):139-143.
[3]H. Sumi *et al., International Journal of Fibrinolysis, Thrombolysis and Extracellular Proteolysis* **6**, Supplement 2 (1992).
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採択課題番号 21P9−3　　中性子実験に向けた蛋白質の精製　　プロジェクト
（京大・原子炉）茶竹俊行、森本幸生
（千科大・薬）柳澤　泰任

PR9-4 Large Scale Preparation of Bio-Macromolecules with a Jar-Fermentor System to Deuterium Treatments

T. Chatake, T. Saito, N. Fujii and Y. Morimoto

Research Reactor Institute, Kyoto University

INTRODUCTION: It is very important to treat biological materials into a deuterium condition to decrease incoherent scattering of the hydrogen including an intact living cell under the neutron scattering method. Moreover, a large-scale culture of bacterium is an essential technique for X-ray and neutron diffraction studies. In these experiments, large amounts of purified proteins are necessary. The most popular method for obtaining these proteins is overexpression of proteins in bacteria, yeast or insect cell. In the present study, we accomplished overexpessions of 20S proteasome from a yeast and mitochondrial importing proteins from rat overexpressed in *Escherichia coli* (*E. coli*).

EXPERIMENTS AND RESULTS: 20S proteasome is a one of biggest complex in a living cell, how their components arrange to be whole particle, we hope and try to prepare them in a large-scale preparation with deuterium solution to use neutron beam. 20S proteasome was obtained from yeast (additive tag strain) by 8 L jar-fermentor system in water medium solution. Aeration, circulation, temperature and some nutrients were checked and modified to be high-yield of the yeast. A one course cultivation gives 80 g yeast, and a successive preparation lets us have 3 mg 20S proteasome sample. A deuterium treatment are now in progress for a neutron experiments. And other proteins, OM37 [1], cloned by Prof. Mihara, Kyushu Univ. and gotten, protein was overexpressed in *E. coli*. A construction vector pET-28b(+), which contained oligonucleotides coding OM37 and 6xHis tag and a cleavage site of thrombin at N-terminus, was used to transform a competent cell *Rosetta* 2(DE3)^{pLysS}. The transformed cell was used for a large-scale culture for overexpression of OM37. 100 μl of the transformed cell was preincubated in 100 ml NZCYM medium with 30 mg/ml kanamycin, and then used for large-scale cultures. The first large-scale culture was carried out using 10 baffle-flasks (1 liter volume for each) in a shaking incubator. Each flask contained 250 ml NZCYM medium with kanamycin. *E. coli* was preincubated at 310K with shaking (190rpm). When ABS_{600nm} of the medium reached at 0.6, the overexpression of OM37 was inducted by 2 μM IPTG. After a 2-hour induction, *E. coli* was harvested. The second large-scale culture was carried out in a Jar fermentor (10 liter volume). 150 ml medium including the transformed cell was preincubated overnight at 310K, then it was added in 9 L NZCYM medium with kanamycin in the Jar fermentor. After 1.5 hour incubation at 310K with aeration, ABS_{600nm} of the medium was 0.827. 2 μM IPTG was added for the induction of the overexpression of OM37. After 3-hour incubation at 310K, *E. coli* was harvested.

The harvested *E. coli* was sonicated on ice, and soluble OM37 protein was separated by ultra-centrifuge (30,000 rpm, 277K, 30 min.). The supernatant was further purified by affinity chromatography. Ni-chelating column was selected for separating this His-tagged protein. Chelating Sepharose Fast Flow liganded Ni^{2+} ion in a XK16/20 column was equilibrated with a buffer containing 20 mM imidazole, 20 mM Tris-HCl (pH7.5), 0.6 M NaCl, 10% glycerol and 1 mM PMSF, then the supernatant was injected. The elution was performed by a gradient of the concentration of imidazole from 20 mM to 500 mM. The affinity chromatography repeated twice in order to obtain highly purified OM37 protein.

In the present large-scale culture of *E. coli*, 11.67 g *E. coli* was obtained from 2.5 L incubation in a shaking-incubator (4.67 g/L). On the other hand, only 25.5 g *E. coli* was obtained from 9 L incubation from a Jar fermentor (2.83 g/L), although the incubation time changed from 2 hours to 3 hours. Moreover, OM37 obtained from the Jar fermentor was partially digested by proteinases. In the present experiments, the large-scale culture by a shaking incubator was more suitable for the overexpression of this protein. Fig.1 shows 15% SDS electrophoresis of the purified OM37 protein obtained from a shaking incubator. A single peak observed in the lane indicated that the overexpression in the large-scale culture and purification by affinity chromatography was successfully accomplished.

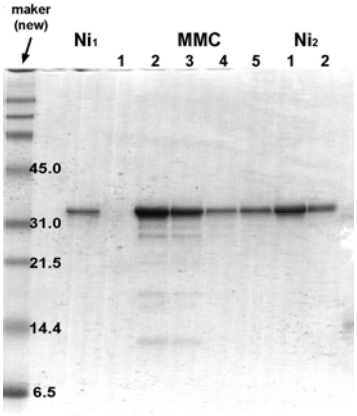

Fig. 1. 15% SDS electrophoresis of OM37. A lane of Ni_1 is OM37 purified by the first Ni-chelating column. Lanes of MMC 1-5 are purified by MMC column after the first Ni-chelating column. Lanes of Ni_2 1-2 are purified by the second Ni-chelating column after the first Ni-chelating.

REFERENCES:

[1] T. Komiya and K. Mihara, *J.B.C.*, **271** (1996) 22105-22110 .

採択課題番号 21P9-5　　重水素化蛋白質調整の大量培養法の技術開発　　プロジェクト

（京大・原子炉）齋藤　毅、藤井紀子、森本幸生

PR9-5 **Crystallization of Ribonuclease A and H/D Exchange for Neutron Diffraction Experiment**

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[1]Japan Atomic Energy Agency

INTRODUCTION: Techniques to exchange hydrogen to deuterium (H/D exchange) are crucially important in neutron diffraction experiments. Deuteration of a crystal of macromolecules is necessary for neutron protein crystallography in order to diminish background noises due to incoherent neutron scattering from hydrogen atoms in the crystal. In the present study, we carried out crystallization of ribonuclease A (RNase A) and H/D exchange of the crystals of RNase A, in order to establish an application of H/D contrast technique on neutron protein crystallography. RNase A is the most popular enzyme in a ribonuclease family. It is widely used in molecular biology and biochemistry, and a lot of structural studies of this protein has been reported, including neutron crystallographic studies of RNase A and its complex with polynucleotides [1.2]. We obtained very huge crystals of RNase A from undeuterated crystallization solution, and performed H/D exchange of the crystal using vapor diffusion technique.

EXPERIMENTS: RNase A was purchased from Wako Chemical Co. Crystallization of RNase A was carried out without further purification because this protein was highly pure. In the first attempt of crystallization, polyethyleneglycol was used as a precipitant, because H/D exchange would not affect crystal growth and crystal structure when this reagent was used as a precipitant for another protein; proteinase K before [3]. Good prism-like crystals appeared in the crystallization solution within 3 days. However, since the nucleation of the crystal was too fast to control, large crystals could not be obtained even after the optimization of the crystallization condition. The interfacial diffusion technique was applied in the second crystallization attempt because it was reported that monoclinic ($P2_1$) crystals of RNase A was obtained in a capillary using this technique [4]. In the present study, we applied this technique with a large scale. 8, 16, 24 and 32 mg of RNase A were dissolved respectively in 400 µl H_2O buffer containing 0.1 M sodium acetate (NaAc) (pH5.7). These solutions were then put into quartz capillaries (ϕ=6mm) for NMR measurements, and frozen for 30 min in –40-degree freezer. Finally, 400 µl 2-propanol cooled at 4 degree was gently added on each frozen protein solution. The crystallization solutions were incubated at 20 degree.

RESULTS: Large crystals of RNase A with the space group of $P2_1$ could be obtained by the interfacial diffusion technique. Crystals appeared after several months' incubation, and the growth of the crystal was terminated after 1 year. The maximum size of undeuterated crystal of RNase (RNase-H_2O) was 2 x 3 x 4 mm^3. This crystal was grown at the bottom of a capillary, so this capillary was cut into 30 mm length, and sealed with the harvest buffer solution (40mg/ml RNase, 0.1M NaAc (pH5.8) and 50% 2-propanol) in order to prevent desiccation of the crystal. This crystal was used for neutron experiments.
Unfortunately, this method could not be applied to deuterated crystals of RNase A (RNase-D_2O) because the nucleation of the crystal was very slow (5 months), crystal appeared at the middle of capillary, which was difficult to handle, and the maximum size of the crystals obtained was 1 x 1 x 2.5 mm^3. The reason would be deuteration of solvent (D_2O) and precipitant (2-d8-propanol, Isotec Inc.). Therefore, we produced a huge RNase-D_2O enough for neutron experiment by deuteration of RNase-H_2O by the vapor diffusion technique. A 40 mm-length capillary including RNase-H_2O at its bottom was turned upside down, and the end of the capillary was soaked in D_2O solution containing 40mg/ml RNase A, 0.1 M NaAc and 50% 2-d8-propanol as shown in Fig. 1.

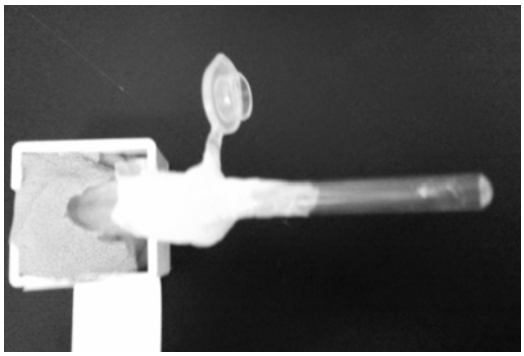

Fig. 1. The photograph of the H/D exchange system of the undeuterated RNase A crystal.

Neutron diffraction experiment of the RNase-H_2O was carried out using the BIX-3 diffractometer at the JRR-3M reactor in Japan Atomic Energy Agency [5]. Neutron diffraction data enough to determine the three dimensional structure of this crystal could be collected in two weeks. Neutron diffraction data collection of the RNase-D_2O is scheduled in the first half of FY2010.

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[5] I. Tanaka *et al.*, *J. Appl. Cryst.* **35** (2002) 34-40.

採択課題番号 21P9−6　緑藻培養法による重水素化炭素源の創出に関する技術開発　プロジェクト
（京大・原子炉）茶竹俊行、森本幸生
（原子力機構・量子ビーム）藤原　悟

PR9-6 Structural Investigation of the 20S Proteasome and Anesthetic Agents

Y. Morimoto, K. Nishio, U. Bahudin[1] and I. Hisatome[1]

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INTRODUCTION: Proteasomes (or multicatalytic protease complexes) are widely distributed in eukaryotes, ranging from human to yeasts. They are involved not only in selective destruction of short-lived regulatory proteins but also in the removal of abnormal, misfolded or improperly assembled proteins generated in cells. There are growing lines of evidence addressing the importance of proteolysis mediated by proteasomes and their partner ubiquitin, which is responsible for many biological processes, including cell cycle, apoptosis, signal transduction, metabolic regulation, and stress response. In addition, in higher eukaryotes, 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.

Proteasomes have molecular masses of approximately 750 kDa and sedimentation coefficients of approximately 20S. They are barrel-like particles formed by the axial stacking of four rings made up of two outer α-rings and two inner β-rings, being associated in the order of αββα The catalytic β-type subunits are located in a chamber formed by the centers of the abutting β rings and the α subunits form a physical barrier for substrates to reach the active sites.

Inhibition of the proteasome activities and degradations could induce anticarcinogenic effects against myeloma cells. Some of serine protease inhibitors are well-known as a protease, but they have not show characteristics of the anticarcinogenicity. One of authors well found anesthetic agents inhibit the proteasome degradate activities, and its schematic drawing is the followings (Fig.1):

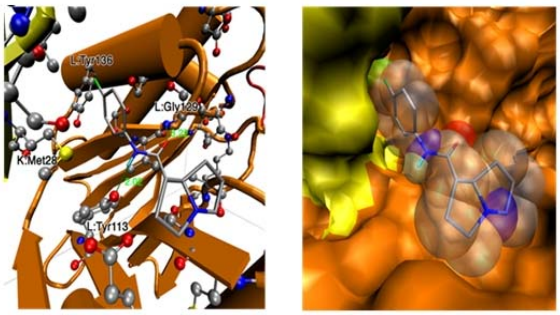

Fig. 1.Schematic drawings of the binding site at lidcaine(left) and spatial packing model(right).

RESULTS & DISCUSSION: In order to clarify atomic information around drugs on the protein structure, we prepare a whole 20S proteasome particle from yeast whose subunit was modified to robust by genetic modification. Yeast (YYS676 strain) was cultured by 10L jar-fermentor was used, and then sample was isolated by an affinity separation with 3-FLAG tags. An yield of the 20S proteasome was 5 mg/8 L culture. Furthermore, a preparation of a deuterium particle was tried by a heavy water usage with slightly modified jar-fermentor cultivation.

Crystallization of the sample was carried out by a vapor diffusion technique with some precipitant reagents, and the following crystals were obtained (Fig.2).

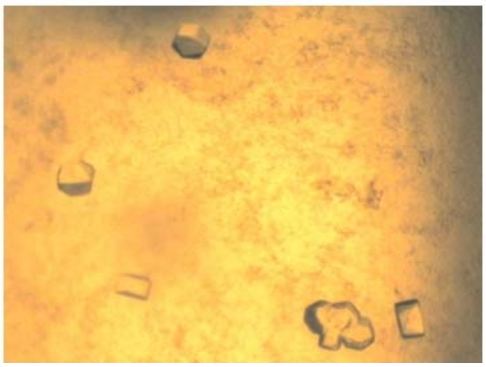

Fig. 2. Crystals of the 20S Proteasome from a yeast.

Crystals were checked and used for X-ray data collection at Spring-8 BL44XU station. The whole structure presents clearly an electron density map, but a drug pocket has no expected map (Fig.3).

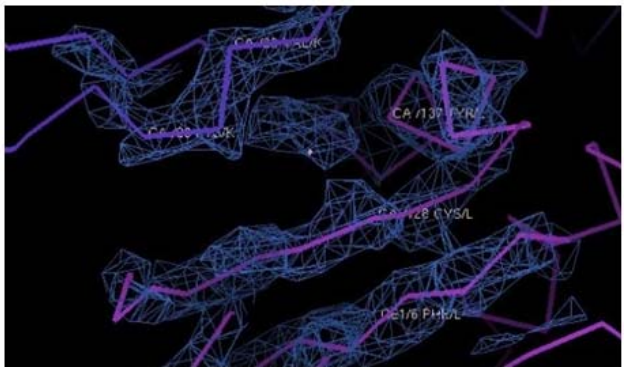

Fig.3 . An electrom density map of the 20S proteasome.

The result leads us to more crystallization with various concentration of the drugs. In contrast, deuterated drugs could detect its positional coordinates in the 20S particle by a neutron scattering method.

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