VIII-Ⅱ-1. Project Reserch

Project 6

PR6 Deuterium Exchages in the Biological Macromolecules for a Neutron Analysis

Y. Morimoto

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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:

22P6-1

Y.Morita, K.Nishio, M. Sugiyama and Y.Morimoto are reported "A liberal structure model of yeast 26S proteasome by small-angle scattering analysis", in which small angle technique creates a visible structure of the 26S large particle in a solution, and the model without other structural information re-produce a actual model by electron microscopy. We will try a formation and combnation of such particle or subunits under various condition of the solution.

22P6-2

Y. Yanagisawa, T. Chatake, K. Chiba-Kamoshida, S. Naito, T. Ohsugi, H. Sumi, I. Yasuda, Y. Morimoto reported "Strutucure Determination of Nattokinase from *Bacillus Subtilis Natto* ". Needle-like crystals of NK were obtained from crystallization solution containing 6.4 mg/ml NK, 50 mmol/l Tris-HCl buffer (pH 7.5), 5 mmol/l $CaCl_2$, 100 mmol/l NaCl, 50 mM HEPES, 5% polyethylene glycol 8000 and 4% ethylene glycol. The crystal belongs to monoclinic *C*2 and its cell parameters are a = 74.3 Å, b = 49.9 Å, c = 56.3 Å and β = 95.2 °. Results show an active amino acid Ser221 and the electron densities. Hydrogen atoms are essential to more active assay as prophylactic and curative medicines, deuterium treatment will start in such crystals.

22P6-5

T. Chatake, S. Fujiwara, T. Ishikawa, Y. Yanagisawa, T. Yamada, I. Tanaka, Y. Morimoto reported "High resolution X-ray crystallographic investigation of the effect of deuteration of crystallization solution" of the result "Crystals of proteinase K were prepared in differently deuterated crystallization solutions, and non-deuterated and deuterated crystal structures were determined at 1.1 Å resolution." It describes "X-ray experiments of crystals in different solutions revealed that there was a close similarity between three-dimensional structures of non-deuterated PK crystal and deuterated PK crystal. Differences between the two structures were very small; main chain and side chain r.m.s.d. values were 0.05 Å and 0.07 Å, respectively, and they correlated well to the B-factor. These observations suggested that no systematic differences exist between deuterated and non-deuterated crystal structures."

22P6-6

Y.Morimoto, K.Nishio, T.Maekawa, H.Yamaguchi and I.Hisatome reported "Structural insights of inner pockets in the yeast 20S Proteasome particle binding inhibitor reagents as anticancer functionings" of the results "The inhibitor has chymotryptic-like activities, inside wall of a b5 subunit is available for such binding mechanism. Actually, TYR(135) and GLY(128) of the subunit interact with -OH and -C=O groups of the inhibitor, but VAL(129) interferes with such interactions. A multiple myeloma cell yields large amounts of immunoglobulin, and the proteasome functions against the globulin molecules, so such interaction is hindered by the proteasome with the inhibitor.".

22P6-3,6-4

N.Fujii has prepared a construct of the human crystalline and obtained small amounts of the sample. Characteristics of the sample by use of mass spectroscopy are now in progress. And T.Saito has tuned available fermentor and now is testing.

採択課題番号 22P6　　生体試料解析のための重水素化手法の確立　　プロジェクト

（京大・原子炉）森本幸生

PR6-1 A Liberal Structure Model of Yeast 26S Proteasome by Small-angle Scattering Analysis

Y. Morita, K. Nishio[1], M. Sugiyama[1] and Y. Morimoto[1].

Graduate School of Science, Kyoto University
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INTRODUCTION: The 26S Proteasome is a large multiprotein complex involved in the regulated degradation of ubiquitinated protein in the cell. The 26S proteasome is composed of two sub-complexes, the core particle (CP, 20S) and the regulatory particle (RP, 19S). CP is formed by axial stacking of four heptameric rings: two inner β-rings and two outer α-rings. Capping at each end of the CP is the RP that regulates the proteolytic function of the protease core. The RP can be further divided into base and lid subcomplexes.
It has not been the way itself how the 26S proteasome degrades ubiquitinated proteins. We experimented dynamic light scattering (DLS) and small angle X-ray scattering (SAXS) to reveal the degradation mechanism.

EXPERIMENTS: The 26S proteasome was extracted from yeast YYS276 strain by a multi-beads shocker cell disruptor, and purified with Superose6 gel filtration; column volume, flow rate, and fraction size were 24ml, 0.1ml/min, and 0.5ml, respectively. Through the purification, sample was in 50mM Tris-HCl pH 7.5, 100mM NaCl, 10% Glycerol, 4mM ATP, 10mM $MgCl_2$.
ALV DLS/SLS 5000 light scattering system equipped with an ALV 5000 multiple digital correlation with light source from a Uniphase 22mW He-Ne laser (λ=632.8nm) was used for DLS experiments. The experiments were performed at seven different scattering angles (30,45,60,90,120,135, and 150°) at 298K. The sample concentration was 0.7mg/ml.
SAXS data was measured at BL40B2 in Spring8. Scattering data were collected in the momentum transfer, q, range $1.5\times10^{-4}-2.2\times10^{-1}$Å. The sample concentration was 1.1mg/ml. The 26S proteasome modeling was performed using GNOM and DAMIN (D. I. Svergun, 1992 and 1999).

RESULTS: As shown in Fig.1, small-angle scattering intensities were plotted in low q range ($1.5\times10^{-4}-5.0\times10^{-2}$Å), expecting a very large component existing in a solution. We have calculated a liberal structure of the 26S component by SAXS intensities with no constraints in a shape of component. Our 26S SAXS model (Fig.2) reflected the 26S electron microscopy (EM) image. It has revealed that the 26S component was measurable by SAXS, though it was the enormous complex. We will measure mixtures of 26S and ubiqutinated substrates to analyze degradation mechanism.

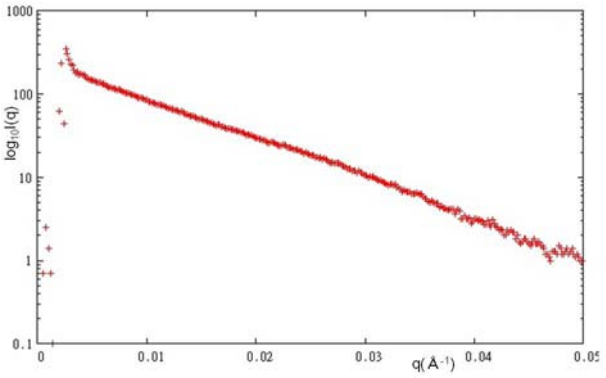

Fig.1. The 26S SAXS data (q: $1.5\times10^{-4}-5.0\times10^{-2}$Å).

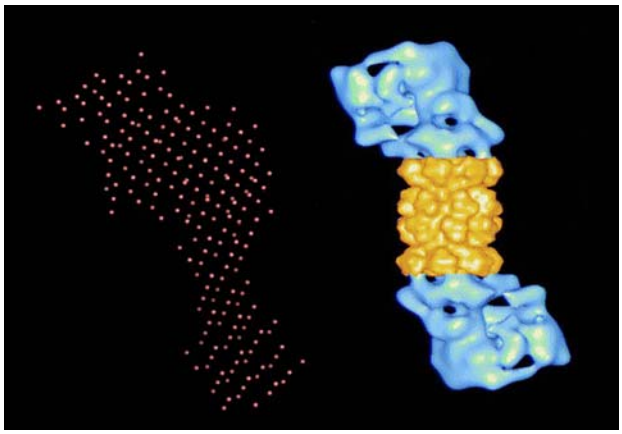

Fig.2. The 26S images; left: our SAXS model, right: the EM image.

REFERENCES:

[1] S. Prekash *et al*., *Nat. Chem. Biol.* **5**, 29 (2009)
[2] L. Bedford *et al*., *Trend Cell Biol.* **20**, 391 (2010)
[3] D. I. Svergun, *J. Appl. Cryst.* **25**, 495 (1992)
[4] D. I. Svergun, *Biophys. J.* **76**, 2879 (1999)

採択課題番号 P6-1　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

PR6-2 Structure Determination of Nattokinase from *Bacillus Subtilis Natto*

Y. Yanagisawa, T. Chatake[1], K. Chiba-Kamoshida[2], S. Naito[3], T. Ohsugi[3], H. Sumi[3], I. Yasuda and Y. Morimoto

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[3]Kurashiki University of Science and Arts

INTRODUCTION: Natto is a popular traditional food in Japan, and is made from soybeans fermented by *Bacillus subtilis natto*. Nattokinase (NK) is a single polypeptide chain composed of 275 amino acids, and has strong fibrinolysis activity [1]. Therefore NK is used as a dietary supplement, mainly in Japan, as both a prophylactic and curative medicine. The homology of NK to subtilisin E (SE) of serine proteases from *Bacillus subtilis* is 99%, and the three-dimensional structure of NK can be predicted based on the crystal structure of SE [2,3], but the X-ray crystal structure of NK is not available yet. Structural information may help elucidating the molecular mechanisms of strong fibrinolytic properties, which are not yet understood. We have recently found that native NK extracted from *Bacillus subtilis natto* may be modified by glycosylation or other chemical reactions [4]. In the present study, purification, crystallization, and preliminary X-ray experiments were carried out toward better understanding of this protein.

EXPERIMENTS: Powder of NK supplied by Honda Trading C. C. was purified by gel filtration chromatography. The diluent was removed using an initial gel filtration on GE Sephadex G10, and the protein solution was then incubated for 12 hours at 277 K in a solution containing 2 mmol/ l PMSF, in order to decrease the activity of self-digestion of NK. After the incubation, NK solution was further purified by a second gel filtration using GE Sephacryl-S200, The purified NK solution was concentrated using centrifugal filtration for crystallization. A solution containing 6.4 mg/ml NK-GC, 50 mmol/l Tris-HCl (pH 7.5), 5 mmol/l $CaCl_2$, and 100 mmol/l NaCl was used for crystallization using Crystal Screens 1 and 2. Crystallization was carried out at room temperature using the sitting-drop vapor diffusion method. 4 mmol/l Pefabloc SC (Roche Corp.) was added to the droplets to avoid self-digestion.
Needle-like crystals of NK were used for X-ray diffraction studies. A preliminary X-ray experiment was carried out using a synchrotron X-ray source. The crystal was soaked in a solution containing 50 mmol/l HEPES (pH 7.5), 5% polyethylene glycol 8000, 4% ethylene glycol and 50% glycerol for 5 seconds. After soaking, the crystal was flash-frozen using 100K N_2 gas, and X-ray diffraction data was collected using synchrotron radiation at SPring-8 facilities (BL44XU). Details of purification, crystallization and X-ray experiment of NK were previously reported [5].
Initial phases of diffractions were determined by the molecular replacement method using the program AMoRe [6], where SE was used as an initial model (1SCJ). Structural refinement was carried out using the program Phenix [7] and Coot [8].

RESULTS: As shown in Fig. 1, needle-like crystals of NK were obtained from crystallization solution containing 6.4 mg/ml NK, 50 mmol/l Tris-HCl buffer (pH 7.5), 5 mmol/l $CaCl_2$, 100 mmol/l NaCl, 50 mM HEPES, 5% polyethylene glycol 8000 and 4% ethylene glycol. The crystal belongs to monoclinic *C*2 and its cell parameters are a = 74.3 Å, b = 49.9 Å, c = 56.3 Å and β = 95.2 °. X-ray diffraction data set was collected at 1.74 Å resolution with the completeness of 69.8 %. R_{merge} of the merged diffraction data was 5.2%. Structural refinement was finished with its *R*-factor (R_{free}) of 13.3% (19.9%). An X-ray density map shows that a PMSF molecule makes a covalently bond to the hydroxyl group of Ser221, which participates in the catalytic triad of this enzyme.

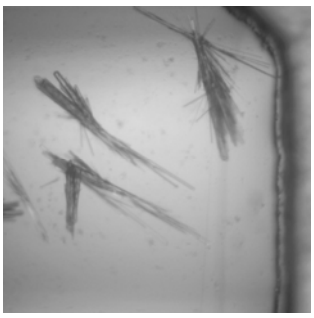

Fig. 1. A photograph of needle-like crystals of NK.

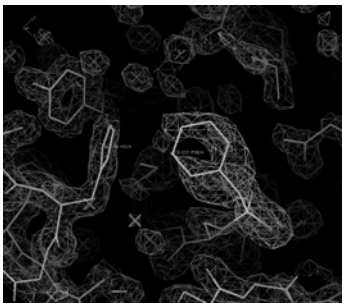

Fig. 2. An X-ray map around Ser221.

REFERENCES:

[1] H. Sumi *et al.*, Fibrinolysis **6**, Suppl. 2, (1992) 86.
[2] Z. Zhng *et al.*, Biochem. J. **395** (3006) 509-515.
[3] Z. Zhng *et al.*, J. Mol. Graphics Modell. **23**, (2004) 373-380.
[4] K. Chiba-Kamoshida *et al.*, Seibutsu Butsuri **50** (supplement 2) (2010) S48.
[5] Y. Yanagisawa *et al.*, Acta Cryst. F**66** (2010) 1670-1673.
[6] J. Navaza, Acta Cryst., A**50** (1994) 157-163.
[7] P. D. Adams *et al.*, Acta Cryst. D**66** (2010) 213-221.
[8] P. Emsley *et al.*, Acta Cryst. D**66** (2010) 486-501.

採択課題番号 22P6-2　　中性子実験に向けた蛋白質の精製　　プロジェクト
（千葉科大・薬）柳澤泰任（京大・原子炉）茶竹俊行、森本幸生

PR6-3 High-resolution X-ray Crystallographic Investigation of the Effect of Deuteration of Crystallization Solution

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INTRODUCTION: Deuteration of macromolecules is an important technique used in neutron protein crystallography. Solvent deuteration of protein crystals is carried out by replacing water (H_2O) with heavy water (D_2O) prior to neutron diffraction experiments, in order to diminish background noises. We investigated the effects of solvent deuteration on the crystallization of proteinase K (PK) with polyethylene glycol as precipitant, using high-resolution X-ray crystallography. PK is a powerful protease, which has highly activities in various environments [1,2]. It is easy to obtain high-quality crystals of PK from *Tritirachium album*, therefore this protein is a good sample for evaluating effect of deuteration to crystallization precisely [3]. In previous studies, eight NO^{3-} anions were included in the PK crystal unit cell, grown in $NaNO_3$ solution. In this study, however, the PK crystal structure did not contain NO_3^- anions, and consequently, we avoided problems of distortions of amino acids due to the presence of NO_3^- anions.
High-resolution X-ray diffraction experiment was carried out in order to investigate effect of deuteration to crystal structure. Crystals of PK were prepared in differently deuterated crystallization solutions, and non-deuterated and deuterated crystal structures were determined at 1.1 Å resolution.

EXPERIMENTS: Crystallization and X-ray diffraction experiment was reported previously [4]. Since we found some biases in PK structures, which were determined in our previous study, we re-determined the structures. Crystal structures of non-deuterated and deuterated PK (hPK and dPK, respectively) were determined by the molecular replacement method [5]. The initial model was the crystal structure of PK, solved at 0.98 Å resolution, obtained from H_2O solution using $NaNO_3$ as precipitant [3]. Structural refinement and model-building were carried out with the program Phenix program [6] and the program Coot [7].

RESULTS: Atomic coordinates of hPK and dPK were determined at 1.1 Å resolution with *R*-factor (R_{free}) of 11.8% (13.2%) and 14.7% (16.2%), respectively. hPK and dPK are very similar to each other. Fig. 1 shows the r.m.s.d for the main chain and side chains of hPK and dPK. The averaged r.m.s.d values for the main chain and side chain are 0.05 and 0.07 Å, respectively. Coordinate errors estimated using the maximum-likelihood method were 0.10 Å for hPK and 0.12 Å for dPK, hence the structural differences between the two PK protein structures were mainly due to coordinate errors in structure determinations. Of 380 hPK H_2O molecules which were observed in the solvent region, 373 (98%) were conserved as dPK D_2O molecules. The averaged r.m.s.d. value of water molecules between hPK and dPK was 0.33 Å, and only 12 (3%) H2O molecules were greater than 1 Å away from the corresponding D_2O molecules.

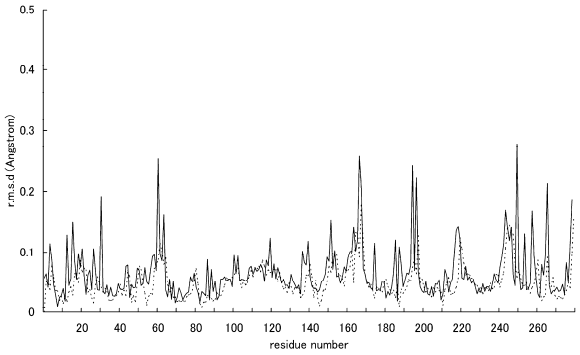

Fig. 1. Root-mean-square differences between hPK and dPK. Solid and broken lines represent the main chain and side chains, respectively.

We obtained high-quality PK crystals from crystallization in polyethylene glycol. X-ray experiments of crystals in different solutions revealed that there was a close similarity between three-dimensional structures of non-deuterated PK crystal and deuterated PK crystal. Differences between the two structures were very small; main chain and side chain r.m.s.d. values were 0.05 Å and 0.07 Å, respectively, and they correlated well to the B-factor. These observations suggested that no systematic differences exist between deuterated and non-deuterated crystal structures. We conclude that polyethylene glycol would be a good precipitant for deuterated crystallization in preparation for neutron protein crystallography experiments, since the effects of deuteration on this crystallization system are small.

REFERENCES:

[1] P. J. Sweeney and J. M. Walker, Meth. Mol. Biol. **16** (1993) 305-311.
[2]W. Ebeling *et al.* Eur. J. Biochem. **47** (1974) 91-97.
[3]C. Betzel *et al.*, Biochemistry **40** (2001) 3080-3088.
[4]T. Ishikawa *et al.*, KURII Progress Report (2009) PR9-2.
[5] J. Navaza, Acta Cryst., A**50** (1994) 157-163.
[6] P. D. Adams *et al.*, Acta Cryst. D**66** (2010) 213-221.
[7] P. Emsley *et al.*, Acta Cryst. D**66** (2010) 486-501.

採択課題番号 22P6-5　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
（原研）藤原 悟、（京大・原子炉）茶竹俊行、森本幸生

PR6-4 Structural Insights of Inner Pockets in the Yeast 20S Proteasome Particle Binding Inhibitor Reagents as Anticancer Functionings

Y. Morimoto, K. Nishio, T. Maekawa[1], H. Yamaguchi[1]and I. Hisatome[2]

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[1]School of Science and Technology, Kwansei Gakuin University
[2]Medical School, Tottori University

INTRODUCTION: Proteasomes 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 inhibitors are well-known as a protease, but they have not shows characteristics of the anticarcinogenicity. One of authors well found anesthetic agents inhibit the proteasome degradate activities. We have tried a three-dimensional structure analysis by crystal structure analysis with reagents coupled crystallization.

EXPERIMENTS AND RESULTS: Yeast 20S proteasome tagged with affinity peptides was prepared by overexpression in yeast. Cells were homogenized by glass beads and the crude extracts purified by M2 affinity chromatography and Mono-Q anion exchange one. 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 Rayonix MX-225HE CCD detector installed in BL44XU, SPring8, Japan. These images were processed by using HKL2000, intensity data up to 2.5 Å become available. Initial phases were determined by molecular replacement method, and the structure model without ligand was refined by *Refmac*.

Whole structure of the 20S proteasome is shown in Fig.1, where depicted clipped particle to show inside wall of the particle. Three-dimensional localization of amino acid residues around S1 pocket is available for one inhibitor. The inhibitor has chymotryptic-like activities, inside wall of a β5 subunit is available for such binding mechanism. Actually, TYR(135) and GLY(128) of the subunit interact with -OH and -C=O groups of the inhibitor, but VAL(129) interferes with such interactions. A multiple myeloma cell yields large amounts of immunoglobulin, and the proteasome functions against the globulin molecules. Such interaction could be hindered by the proteasome with the inhibitor, apoptosis of cancer cells will be induced under treatments.

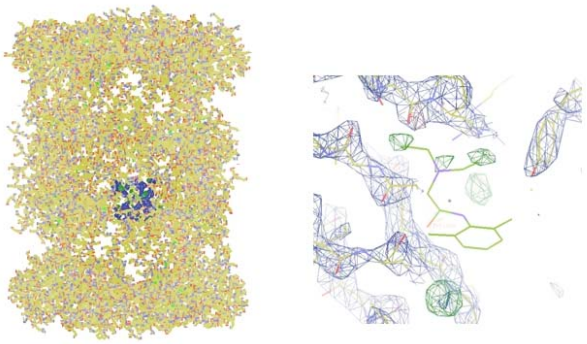

Fig.1. Structures of the yeast 20S proteasome:
whole (left) ; electron densities and inhibitor compound (right)

Preparation of more active reagents designed by structural coordinates for some other binding sites and analysis of its inducing mechanisms of apoptosis have just started.

採択課題番号 22P6-6　酵母プロテアソームの高次構造形成と活性動作機構の解明　プロジェクト
（京大・原子炉）森本幸生（鳥取大医）久留一郎、Udin Bahudin
（関学理）山口 宏、前川琢磨、細川桂一（茨城大フロンティア）海野昌喜、小原裕二