VIII-Ⅱ-1. Project Research

Project 2

PR2 Deuterium Exchanges 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) Development of deuterium carbon sources, S.Fujiwara
(4) Deuterium DNA, and Protein purification , T.Chatake
(5) A new enzyme usable for a low carbon energy system, Y.Hidaka

There are the following research activities in this year.

RESULTS:

23P2-1

Y. Morita, K. Nishio, T. Inobe, M. Sugiyama and Y. Morimoto are reported "A whole structure models of yeast 26S and 20S proteasome by small-angle scattering", in which small angle technique creates a visible structure of the 26S large particle in a solution, and the model without any structural information is re-produced as an actual model. Futhermore, a degradative substrate (ubiquitinated protein) is planned and expressed in *E.coli,* then a purification has been started. They are trying to experiments with X-ray or neutron scattering of a mixture among 26S and substrate proteins under various condition of the solution. The same condition is also used for an atomic force microscopy (AFM).

23P2-2

T. Chatake, Y. Morimoto, Y. Yanagisawa reported "Cultivation of *Bacillus subtilis natto* in deuterated medium". The previous work in the last proposal reported a natto-kinase crystallization. The crystal structure has been determined, and futher work will start as a neutron analysis. So a deuterated sample is essential for the work, they appilied a cultivation of cells with deuterium medium. A newly finding has been opend. *Bacillus subtilis natto* strain is suitable in a deuterium medium lather than a general (hydrogen:H_2O) solution. The result is described in this report with cell growth curvature.

23P2-3

T. Chatake, S. Fujiwara , A. Kawaguchi, T. Matsuo , Y. Morimoto reported "Measurements of neutron transmissions of biomacromolecular solutions using 4CND" of the result for the 4CND in KUR B3 beal line. Transmissions of biomacromolecular are essential and critical against a neutron diffraction experiments, a pre-check diffraction or transmission should be done with monocromatic neutron beam. The result explained as "The present experiments confirmed that 4CND can be used for measuring neutron transmission of macromolecular solutions for several minutes per sample. Assuming that 4CND was applied for neutron transmission of macromolecular crystal (~8 mm^3), efficiency of data collection would be 1/200. If accuracy becomes 1/10, the experimental time for one crystal is estimated to be 1.5 hr. ".

23P2-4

Y. Hidaka, Y. Morimoto, H. Yamaguchi, Y. Nozu, K. Hosokawa, T. Maekawa, A. Ukawa, J. Ishibashi, S. Shimamoto reported "High Efficient Lignin- Degradation System of *Pleurotus Eryngii* Versatile Peroxidase VPL2" focusing to a low carbon energy world. They said the results "We found that the recombinant VPL2 was well expressed as a soluble form in T7 Shuffle cells which over-expresses DsbC protein. Therefore, the recombinant protein was purified with several types of chromatography. The enzyme assay of the recombinant VPL2 was in progress.

23P2-5

Y.Morimoto, T.Maekawa, K.Nishio, I.Hisatome and H.Yamaguchi reported "Structural Insights of Anesthetic Agents in the S1 Pocket of Yeast 20S Proteasome". A newly inhibitor of the 20S proteasome, so called a anesthetic agent, binds to the S1-pocket inside the whole 20S particle. The compound is packed among two or three β-strand of the 20S proteasome, and interacts with some amino acids of this 20S proteasome.

PR2-1 Whole Structure Models of Yeast 26S and 20S Proteasome by Small-Angle Scattering

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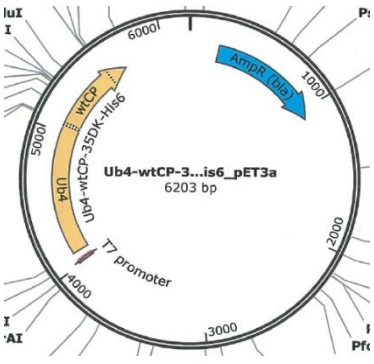
INTRODUCTION: The 26S Proteasome is a large multiprotein complex involved in the regulated degradation of ubiquitinated protein in the cell. The 26S proteasome is composed of two sub-complexes, the core particle (CP, 20S) and the regulatory particle (RP, 19S). CP is formed by axial stacking of four heptameric 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. Biological function of degradation within the 26S proteasome and substrate molecule is very important and interesting to clarify a process, a substrate composed of ubiquitin and protein should be supplied. We experimented atomic-force microscopy (AFM) and small angle X-ray scattering (SAXS) to reveal the degradation mechanism when we have prepared ubiquitin fused protein expressed by *E.coli*.

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 $MgCl_2$.



Ubiquitin fused protein was expressed in BL21 Rosetta / pET3a vector designed as 6-HisTag + 4Ub + 35kDa protein. 1.8L cultivation and harvesting cells were carried out and we have obtained 1.8 g of *E.coli* cells.

A Ni-affinity column chromatography is available for a purification of the Ub4 substrate. A mixture of 26S proteasome and Ub4 substrate with 1:1 molar ratio is checked and measured in enzymatic activities. Ub4 substrate may be replaced in deuterium solution for a neutron scattering measurement, but it is not applied in this time.

SAXS data was measured by RIGAKU Bio-SAXS on 1.0 mg/ml protein concentration with MicroMax X-ray generator. The 26S and 20S proteasome modeling were performed using GNOM and DAMIN (1,2).

RESULTS: A fig.1 shows schematic representation of an interaction among 26S, 20S, UCH37 and Ub4 substrate, preliminary model analyses of 26S and 20S whole particles are succeeded in this work.

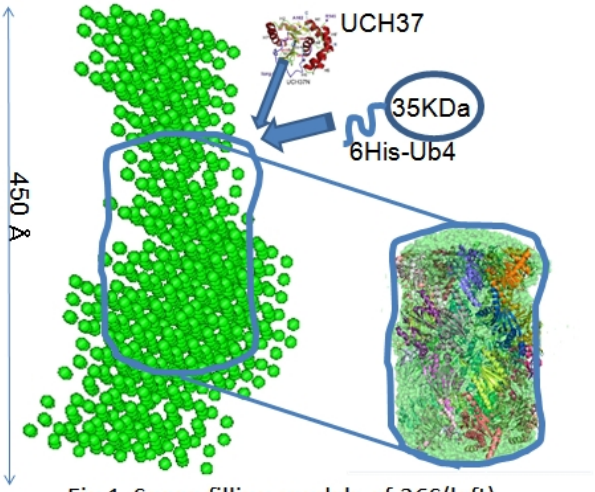


Fig.1 Space-filling models of 26S(left) and 20S(right) proteasomes from SAXS: 20S located in the center of 26S particle: 20S model superimposed by crystal structure.

The 26S proteasome has a 20S core in the center of the whole particle and acts with a substrate of Ub4, when the UCH 37 enzyme recover digested ubiquitins to recycle them. SAXS will show the process of such a degradation system with two or three actors in an aqueous solution.

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[2] D. I. Svergun, Biophys. J., **76** (1999) 2879.

PR2-2 # Cultivation of *Bacillus subtilis natto* in Deuterated Medium

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INTRODUCTION: Deuteration of protein is an important technique in neutron biology. It is essential for the contrast-variation method in neutron small angle scattering, and improves quality of diffraction data in neutron crystallography, because of higher atomic scattering length of deuteriumthan hydrogen. In the present study, *Bacillus subtilis natto* was cultured in deuterated medium in order to obtain deuterated samples of nattokinase (NK) and menaquinone 7 (MK-7), which this bacterium secretes in culture medium during cultivation. NK belongs to a family of serine protease, and has strong fibrinolytic activity [1]. In addition, NK activates other fibrinolytic enzymes such as pro-urokinase [2] and tissue plasminogen activator [3]. Currently, NK is used as a dietary supplement, mainly in Japan, as both a prophylactic and a curative medicine. Recently, we determined the non-hydrogen structure of NK by X-ray crystallographic analysis [4]. MK-7 is a large bimolecular complex (100 - 200 KDa) containing vitamin K_2, which promote ossification. While vitamin K_2 is a lipophilic organic compound, MK-7 is a water soluble complex [5]. Therefore, NK and MK-7 would be useful materials in food engineering and pharmacy. Deuteration of protein was usually carried out using *Escherichia coli*. On the other hand, we used *Bacillus subtilis natto* to obtain deuterated samples of natural NK and MK-7.

EXPERIMENTS: *Bacillus subtilis natto* Miyagino (BSNM) was used in the present study. Culture medium contained 2% polypeptone S and 3% glycerol. Native BSNM was pre-cultivated at 310 K for 2 days in 300 mL non-deuterated medium, and then 20 μL of the pre-cultivated medium was added in 5 mL of 25% deuterated medium, which contained D_2O and H_2O at the ratio of 1:3. Cultivation was carried out 310 K for 7 days in several test tubes, and good BSNM were used for the next successive subculture. Percentage of D_2O gradually increased in the series of the successive subcultures by the step of 25%, and we obtained 100% deuterated BSNM (dBSNM) after seven successive subcultures. Because all cultivation was carried out in open system, solvent exchange from D_2O to H_2O would occur by vapor diffusion during the cultivations. The solvent exchange was assessed by measuring neutron transmission using 4CND at the beamline B3 at Kyoto University Research Reactor. The detail of this neutron experiment and the result is reported in the other report of this KUR Progress Report 2011 (Project No. 23P2-3). Assays of NK and MK-7 in culture medium of dBSNM were carried out after cultivation. The fibrinolytic activity of NK was assessed using the fibrinplate method [6], and the molecular weight was measured using 15% SDS electrophoresis. MK-7 was assessed using HPLC system [7].

RESULTS: Fig. 1 shows growth curves of dBSNM in 0%, 50%, and 100% deuterated culture medium. BSNM made endospores during the growth process, and the conductivity of culture medium temporarily decrease at time of endosporulation. Therefore, the normal growth curve of BSNM took a zigzag. The growth curve of dBSNM in 0% or 50% D_2O medium did not take a zigzag; once the conductivity began to decrease, it never increased again, suggesting that dBSNM could not grow normally in medium containing H_2O. On the other hand, the growth curve of dBSNM in 100% D_2O medium took a zigzag, indicating the success of this cultivation. It can be concluded that dBSNM would be a D_2O specific variant of BSNM. The assays of the 100% D_2O culture medium after the cultivation of dBSNM showed high activity of NK. But, activity of MK-7 was not confirmed. These results suggested that biosynthesis system of MK-7 did not work in dBSNM.

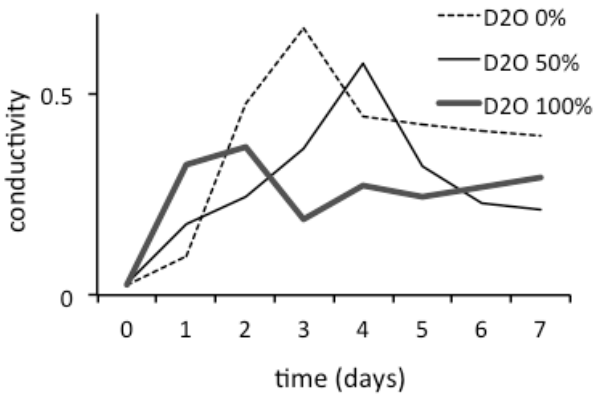

Fig. 1. Growth curves of dBSNM in 0% (a broken line), 50% (a line) and 100% (a bold line).

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採択課題番号 23P2-2　　納豆菌由来蛋白質の重水素化　　プロジェクト
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（千科学大・薬）柳澤泰任

PR2-3 Measurements of Neutron Transmissions of Biomacromolecular Solutions Using 4CND

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INTRODUCTION: 4CND is the four-circle diffractometer at the beamline B3 at KUR. Due to the low intensity of the neutron beam, neutron diffraction of macromolecular crystals cannot be collected using 4CND. However, neutron transmission is expected to be measured, because it does not require intense neutron beam. Neutron transmission of macromolecules can be used as a factor for scaling the data of neutron spectroscopy and scattering. It is also useful for estimation of degrees of deuteration of macromolecular solutions containing many kinds of solutes. In the latter case, neutron transmission would be superior to other methods. For example, Mass spectroscopy cannot measure total exchange from hydrogen atoms to deuterium atoms in all of solvents and solutes at once, and it is necessary to estimate tendency of H/D exchange for each of solvents and solutes individually. On the other hand, neutron transmission of the macromolecular solution directly corresponds to the total H/D exchange of the solution. Two kinds of samples were used for the present study. One was protein-solution in aluminum cells, of which neutron inelastic scattering data were taken at ILL (Samples_NS). Another was culture-medium of *Bacillus subtilis natto* containing heavy water (D_2O) (Samples_CM).

EXPERIMENTS: The Samples_NS corresponds to the aluminum cells containing the sample solutions after the neutron experiments at ILL. They were used without any modification after the neutron experiments. The Samples_CM was prepared at Chiba Institute of Sciences. *Bacillus subtilis natto* were cultured under various ratio of D_2O. The details of the culture were described in the other repot in this Progress Report (Proposal No. 23P2-2). After the culture, the culture medium was centrifuged and then filtered to remove bacteria. These solutions were sealed into capillaries in TL building.
Neutron transmission was measured using 4CND at KUR. Neutron beam was monochromatized at 1.0 Angstrom by a Cu monochromator. Neutron transmission measurements of the samples_NS were performed twice with operating the reactor at 1 MW and 5 MW. The measurements of the samples_CM were done at 5 MW. Exposure times at 1 MW and 5 MW were about 10 min and 4 min, respectively. The exposure time was adjusted using the neutron monitor, which located in-between the monochromator and the sample. Fig. 1 shows one example of samples mounted on the goniometer of 4CND. In this picture, a capillary containing culture medium was mounted on an aluminum mounter, and neutron beam was collimated at the sample position by cadmium sheets.

Fig. 1. Sample NS, on the goniometer of 4CND

RESULTS:
For the Samples_NS, the transmission of the fifteen aluminum cells containing different solutions was measured. Neutron transmission for 4 min at 5MW was almost same as those for 10 min at 1 MW, indicating appropriateness of these neutron experiments. It is probable that even at low intensity of KUR, neutron transmission of protein solution would be helpful for analysis of neutron spectroscopic data of them.
Table 1 shows the result of the transmission measurements of the Samples_CM. Because the culture of B*acillus subtilis natto* was carried out with the open system, there was a risk of exchange from D_2O to H_2O by vapor diffusion. The exchange was assessed by the neutron transmission measurements of the culture medium. The values of the transmission were not different between before and after the culture, and the values of the culture medium in 0% and 100% D_2O were similar to those of pure D_2O and pure H_2O. This suggests that H/D exchange during the culture would be negligible for neutron experiments, and deuteration of solutes would not affect neutron transmission much.
The present experiments confirmed that 4CND can be used for measuring neutron transmission of macromolecular solutions for several minutes per sample. Assuming that 4CND was applied for neutron transmission of macromolecular crystal (~8 mm^3), efficiency of data collection would be 1/200. If accuracy becomes 1/10, the experimental time for one crystal is estimated to be 1.5 hr.

Table 1. Neutron transmission of samples_CM

Culture medium	0% D_2O	50% D_2O	100% D_2O
Neutron transmission			
Before cultivation	61.0	74.9	88.4
After cultivation	61.7	74.2	90.4
Pure D_2O	62.6		
Pure H_2O	92.7		

採択課題番号 23P2-3　生体分子結晶の重水素置換と中性子散乱による検定　プロジェクト
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（原研）藤原悟、松尾龍人

PR2-4 High Efficient Lignin-Degradation System of *Pleurotus Eryngii* Versatile Peroxidase VPL2

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INTRODUCTION:

Lignin peroxidase plays an important role in the biodegradation of the plant cell wall, consisted of lignin. Versatile peroxidase (VP) has been recently reported as a new family of ligninolyic peroxidase, together with lignin-peroxidase and manganese peroxidase. *Pleurotus eryngii* produces a versatile peroxidase which is able to oxidize both Mn^{2+} and aromatic compounds. Thus, the most interesting feature of VP is that it possesses substrate specific characteristics of the other peroxidase families. Therefore, it is very useful to degrade phenolic, non-phenolic lignin dimer, and a numerous aromatic compounds to obtain low molecular organic compounds as an energy resource.

Here we report on the expression of *Pleurotus eryngii* VPL2 using *E. coli* expression system(1,2). The recombinant VPL2 was obtained as a soluble proteins and purified with several types of chromatography..

EXPERIMENTS: The cDNA encoding *Pleurotus eryngii* VPL2 was chemically synthesized and cloned into the pET17b and pET32b expression vectors between the *Nde*I and *Xho*I sites. *E. coli* cells, transformed with the expression vector, were cultured at 37℃ in Luria broth supplemented with ampicillin (50mg/L). After incubation at 37℃ for 10hr, the cells were harvested, washed with 50 mM Tris/HCl (pH7.5), sonicated, centrifuged (15000 x g, 15min), and applied to SDS-PAGE.

RESULTS: In order to express *Pleurotus eryngii* VPL2 in *E. coli* cells, the cDNA fragments encoding VPL2 were chemically synthesized and ligated. Then the full length cDNA was cloned into two type of the expression vector, pET17b and pET32b.

The recombinant VPL2 was expressed as a soluble and an insoluble form using pET32b and pET 17b. Therefore, we first tried to purify the Trx-fusion VPL2 using Ni-chelate and ion exchange chromatography. However, the recovery of VPL2 from chromatography was low and the following enzymatic digestion with thrombin was also insufficiently completed.

To obtain a soluble form of VPL2, we explored proper condition for the incubation of BL21(DE3) cells, transformed with the pET17b expression vector because the recombinant VPL2 was expressed as a insoluble form in BL21(DE3) under a typical condition, as shown by lane 4 in Fig. 1A.

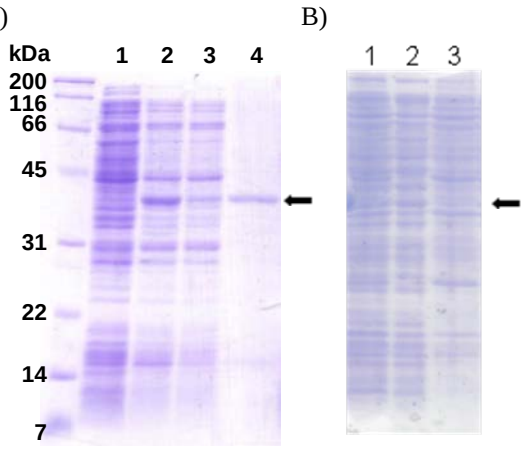

Fig. 1 SDS-PAGE of the recombinant VPL2 expressed in BL21(DE3) (A) and T7 Shuffle (B) cells. A: lane1, *E. coli* cells without the expression plasmid; lane 2, total cells; lane3, supernatant; lane4, precipitates. B: lane 1, total cells; lane2, supernatant; lane3, precipitates.

We found that the recombinant VPL2 was well expressed as a soluble form in T7 Shuffle cells which over-expresses DsbC protein, as shown by lane 2 in Fig. 1B. Therefore, the recombinant protein was purified with several types of chromatography.

The enzyme assay of the recombinant VPL2 was in progress.

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[2] S.Camarero, F.J.Ruiz-Dueñas, S.Sarkar, M.J.Martínez, A.T.Martínez, , *FEMS Microbiol Lett.* **191**, (2000)37-43.

採択課題番号 23P2-4 リグニン分解酵素解明による植物由来有機材料への転換 プロジェクト
（近大）日高雄二、島本 茂、（京大原子炉）森本幸生、
（関西学院大）山口 宏、細川桂一、前川拓摩、野津祐三、鵜川 就、（農生）石橋 純

PR 2-5 Structural Insights of Anesthetic Agents in the S1 Pocket of Yeast 20S Proteasome

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INTRODUCTION: Proteasomes are widely distributed in eukaryotes, ranging from human to yeasts. 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. The 26S proteasome is composed of two 19S regulatory components and a 20S proteasome. 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 ring consists of 7 similar but heterologous subunit. N-terminal tails of α-ring subunit form an entry gate to facilitate passage of substrate. 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. It is necessary to disclose the structural details of S1 active site of 20S proteasome and its complex with the inhibitor. We have analyzed the 20S proteasome from yeast, and are going to study the mechanism or S1-pocket specificities with atomic resolution. Here we report on the preparation of yeast 20S proteasome, the enzymatic activities with inhibitor, crystallization, and also on initial determination of the structure.

EXPERIMENTS: Yeast 20S proteasome tagged with affinity peptides was prepared in yeast. Cells were homogenized by glass beads and the crude extracts purified by M2 affinity chromatography and Mono-Q anion exchange one. Isolated 20S proteasome was concentrated by ultrafiltration, and co-crystallized with small ligand molecule. Vapor diffusion method was applied. Crystals are isomorphous as described in the previous paper and belong to the space group $P2_1$. Initial phases were determined by molecular replacement method, and the structure model without ligand was refined by *Refmac*.

RESULTS: Fig.1 shows structural drawing and chemical structure of the compound.

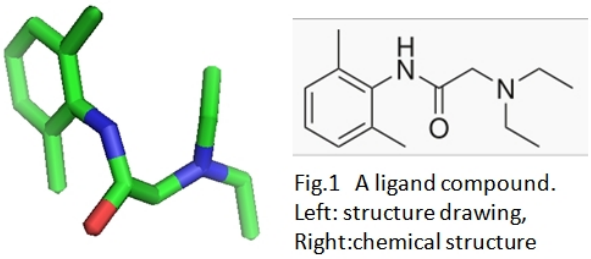

Fig.1 A ligand compound.
Left: structure drawing,
Right:chemical structure

The S1 pocket active site is located inside 20S whole particle (Fig.2 left). 20S whole structure is composed of 28 subunits and has many crevices within neighboring subunit. Ligands are passing through the crevice into an inner-space of the particle. Three-dimensional localization of amino acid residues around S1 pocket is available for one inhibitor; TYR(135) and GLY(128) interact with -OH and -C=O groups, but VAL(129) interferes with such interactions(Fig.3).

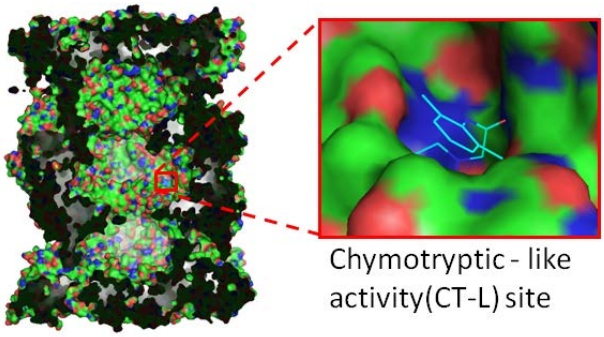

Fig.2 Structure of 20S proteasome and a ligand complex
left: whole 20S proteasome with cross-section,
right: a ligand stick model in space filling S1 pocket of the 20S

Results show possible inhibition with yeast proteasome according to the structural information of bovine one and biological assay of yeast proteasome. We are preparing crystals by means of co-crystallization or inhibitor-soaking crystal method. Analysis of their structure is now under progress.

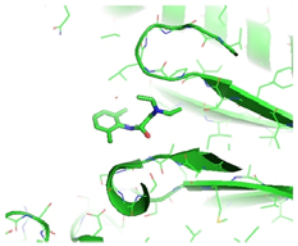

Fig.3 Ligand located in S1 pocket:
ligand compound is stick model and
20S H-subunit ribbon drawing.
The ligand is put on β-strands like scissors

採択課題番号 23P2-5 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、前川拓摩、西尾和也、海野昌喜、小原裕二、Udin Bahudin、久留一郎、山口宏