This presentation will discuss crystallization of human α-thrombin-bivariludin complex and bovine trypsin-BPTI complex, aiming neutron single crystal structure determination to investigate enzyme-inhibitor interactions in terms of hydrogen position and protonation state. Despite neutron diffraction has a great advantage to observe hydrogens, which play important roles in protein activities, the diffraction experiment still needs crystals larger than 1 mm^3 because intensity of neutron beam is limited. To grow big crystals, first, a crystallization phase diagram was drawn for each protein-inhibitor complex. Then, based on the phase diagram, proper crystallization condition was tried and refined. Bovine trypsin-BPTI complex crystal larger than 1 mm^3 can be grown constantly using vapor diffusion method, if the crystallization is started from near the nucleation border on the phase diagram. We are refining the crystallization condition with checking a crystal quality with x-ray diffraction experiments. For human α-thrombin-bivariludin complex, to grow big a crystal needs macroseeding because reproducibility of crystallization is low and the same method as used for bovine trypsin-BPTI complex was not applicable. The crystallization method is as follows: A crystal was seeded at unsaturated region on the phase diagram to prevent other crystals from growing. When the growing stopped, new solution of α-thrombin-bivariludin complex was added. So far, this method gave a crystal with 0.4 x 0.6 x 0.9 mm size. Further effort to improve the crystal size is undergoing.

Keywords: crystal growth, serine protease, neutron single crystal structure determination

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Structural basis for the antiproliferative activity of the Tob-hCaf1 complex

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Cell proliferation and differentiation in multicellular organism are regulated precisely by a variety of proteins that control replication, transcription, translation, and signal transduction. Loss of antiproliferative mechanisms causes abnormal cell proliferation and leads to diseases. Thus, in normal cells, cell cycle progression is tightly regulated by several antiproliferative proteins. The Tob/BTG family is a group of antiproliferative proteins containing two highly homologous regions, Box A and Box B. These proteins all associate with CCR4-associated factor 1 (Caf1), which belongs to the ribonuclease D (RNase D) family of deadenylases and is a member of CCR4 complex. To help elucidate the relationship between the antiproliferative activity of Tob and the degradation of the poly (A) tail, we determined the crystal structure of the complex of the N-terminal region of Tob and human Caf1 (hCaf1). Tob exhibited a novel fold, whereas hCaf1 most closely resembled the catalytic domain of yeast Pop2. Interestingly, the association of hCaf1 was mediated by both Box A and Box B of Tob. Taken together with cell growth assays using both wild-type and mutant proteins, structural studies revealed that complex formation is crucial to cell growth inhibition. The Tob/Caf1 complex serves as a scaffold to tether the poly(A) binding protein and the CCR4 deadenylase complex, thus enhancing the deadenylation efficiency of the poly(A) tail and leading to the suppression of cell proliferation.

Keywords: cell cycle and development, protein interactions, ribonuclease

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Towards the structure of the β4 subunit of the human BK channel

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The BK potassium channel is intimately involved in the regulation of calcium signalling pathways, most notably in providing a negative-feedback mechanism to regulate the activity of L-type-voltage-dependent calcium channels (VDCCs), preventing runaway Ca^{2+} influx. The physiological consequences of this simple regulatory loop are diverse; from vasodilation, to neurosecretion, to neuronal excitability, the BK channel has a variety of physiological roles, each of which requires the channel to have different electrophysiological properties. The phenotypic diversity of the BK channel is mediated by association with a class of tissue-specific transmembrane proteins, the BK β-subunits. These proteins have diverse effects on the molecular properties of the channel. To resolve the ambiguities surrounding the structure and function of the β-subunits, we aimed to determine the structure of the ectodomain of one of these proteins, the β4 subunit of the human BK channel. Here, we report the expression, refolding and crystallisation of the β4 subunit ectodomain and discuss progress towards structure determination. We are producing the β 4 subunit ectodomain by expression in Escherichia coli followed by purification and refolding of the recombinant protein. Crystals were obtained and a complete native dataset collected. Derivatisation with Ta_6Br_{14} resulted in an unusual shift in the symmetry of the crystals - the native crystals were orthorhombic, while the derivatives appeared tetragonal. Attempts to phase the structure are currently underway.

Keywords: potassium channel, calcium signalling, Slo1

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Crystallographic study of extracellular dermal glycoprotein of carrot

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Carrot extracellular dermal glycoprotein (EDGP) may play important role in plant defense systems and in signal transduction. Expression of EDGP is induced by biotic or abiotic stress. The amino acid sequence alignment shows that EDGP shares significant sequence homology with proteins from legumes, tomato, Arabidopsis, wheat, and cotton. Most of the Cys residues in these proteins are conserved. EDGP has six disulfide bonds all Cys residues are involved in disulfide bond and EDGP has four N-linked glycan chains. The glycans and glycosylation in EDGP are essential for defense systems and EDGP secretion. The protein from soybean is termed as leginsuln

binding protein (LBP), which binds insulin and 4-kDa hormone-like-peptide (leginsulin). LBP shows protein kinase activity in vitro and the activity is stimulated by binding of leginsulin. Carrot EDGP and soybean LBP share about 33% sequence homology. Carrot EDGP binds also insulin and leginsulin from soybean in vitro and localizes in the plasma membrane and middle lamellae of cell walls, EDGP also shows protein kinase activity. However, detail about function of EDGP is still unclear. Thus, we work on structural study of carrot EDGP. The structure will provide to a clue to understand the function of EDGP and pave a way for further analyses, which expected new solution for plant defense systems and in signal transduction. EDGP was purified from culture medium of carrot callus by ion exchange chromatography. Crystals of EDGP were obtained by conventional hanging drop vapor diffusion method. The crystal belongs to hexagonal system with cell dimensions of $a = b = 129.8$, $c = 44.4$ Å, and $\gamma = 120^{\circ}$. Structure determination of EDGP is now in progress.

Keywords: extracellular dermal glycoprotein, carrot, crystalazation

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Crystallization of *Clostridium botulinum* serotype D neurotoxin complex

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Clostridium botulinum produces seven immunologically distinct neurotoxin (BoNT; 150 kDa) serotypes, classified as A-G. In culture fluid and naturally contaminated foods, BoNT exists as part of large toxin complexes (TCs) through association with non-toxic non-hemagglutinin (NTNHA; 130 kDa) and three hemagglutinin (HA) subcomponents, HA-70, HA-33 and HA-17 (70, 33 and 17 kDa, respectively). Serotype A-D strains produce M-TC (BoNT/NTNHA complex; 280 kDa) and L-TC (BoNT/NTNHA/HAs complex; 750 kDa), while serotypes E and F strains produce only M-TC. The M-TC is formed first by assembly of a single BoNT and a single NTNHA molecule, and is subsequently converted to the complete L-TC. However, M-TC containing nicked NTNHA at unique site could no longer convert to the L-TC with HA subcomponents. Although the crystal structures of several serotype BoNTs and serotype D HA-33/HA-17 complex[1] have been determined, no structure of BoNT complex forms (M-TC and L-TC) has been demonstrated. In this study, highly purified M-TC was obtained from the supernatant of *C. botulinum* type D strain 4947 through several chromatographic runs. The nicked form of M-TC was prepared by limited trypsin treatment, and was crystallized using the hanging-drop vapor-diffusion technique. The drops consisted of 6 μl protein solution (2.8 mg ml^{-1}) and 4 μl reservoir solution (0.1 M MES pH 6.5, 0.18 M cesium and 15% PEG 6000) derived from condition number 24 of the Hampton Crystal Screen 2. The crystals grew at 293 K and reached dimension of $0.2 \times 0.1 \times 0.05$ mm in 7 days. X-ray data were collected on a Rigaku R-AXIS VII imaging-plate system, using CuKα radiation from a Rigaku FR-E rotating-anode generator. The crystals diffracted to approx. 8Å resolution.

[1]Hasegawa K. et al., J. Biol. Chem. 2007, 282, 24777

Keywords: crystallization, botulinum toxin complex, protein interactions

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Expression and crystallization of *Drosophila* EcR/USP

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The ecdysteroid hormones regulate the major stage of insect development, especially molting and metamorphosis, by binding to a heterodimer composed of the ecdysone receptor (EcR) and the ultraspiracle protein (USP). Even though all insects use ecdysteroid, 20-hydroxyecdysone, as a natural molting hormone, they exhibit different sensitivity for non-steroidal ecdysteroid agonists. The aim of this work is to clarify the molecular mechanism of the functional complexes and the binding mode of non-steroidal agonists to EcR. Here we report the expression and crystallization of EcR- and USP-ligand binding domains, EcR-LBD and USP-LBD, from *Drosophila melanogaster* toward structure solution by X-ray crystallography. EcR-LBD with an N-terminal GST/His tag (GST/His-EcR-LBD) and USP-LBD with a C-terminal His tag (USP-LBD-His) were expressed in E. coli. The expression plasmids for these genes were constructed with two procedures as follows: EcR-LBD and USP-LBD genes were cloned in pET20b and pET41a expression vectors, respectively, and both of EcR-LBD and USP-LBD genes were tandem inserted into pET41a. In the latter procedure, the complex of GST/His-EcR-LBD and USP-LBD-His were expressed in E. coli Rosetta2(DE3)pLysS strain. After purification by affinity and anion-exchange columns, cleavage of EcR-LBD from GST-His was achieved using a site-specific protease, enterokinase. The search of the crystallization condition is currently in progress.

Keywords: ecdysone receptor, *Drosophila*, crystallization

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Molecular basis of histone H3K4ME3 recognition by ING4

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The Inhibitors of Growth (ING) family of tumor suppressors consists of five homologous proteins involved in chromatin remodeling. They form part of different acetylation and deacetylation complexes, and are thought to direct them to specific regions of the chromatin, through the recognition of trimethylated-K4 in the histone-3 tail (H3K4me3) by their conserved Plant HomeoDomain (PHD). We have determined the crystal structure of ING4- PHD bound to H3K4me3, which reveals a tight complex stabilized by numerous