C5 complex.

The titration of B4' with CaM-C5 exhibited signal increase in a similar way as the titration with CaM, implying that the primary binding site of this peptide is N domain of CaM. In contrast the titration of other peptides with CaM-C5 did not exhibit significant signal change, implying that the primary binding site of those peptides are C domain of CaM where C5 occupies already. We further examine the B4' binding site by employing NMR.

2PT146 Characterization of norovirus RNA replicase *in vitro* for the autonomous protein evolution using *in vitro* virus

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We have been trying to construct an autonomous protein evolution system, combining the *in vitro* virus (IVV) and the one-pot isothermal RNA replication reaction. In our system, the norovirus RNA replicase (3D^{pol}) is planned to be displayed on the IVV.

In this work, we evaluated the activity of 3D^{pol}, which was synthesized in cell-free protein synthesis system. As indicated in the previous report, when a ss (single-stranded) RNA was incubated with 3D^{pol}, it was replicated to a ds (double-stranded) RNA in a primer-independent manner. And the isothermal amplification of the dsRNA by eight-fold was realized. In the primer-independent replication reaction, the presence of C-stretch at the 3'-terminus increased the replication efficiency by six-fold. Contrary to the previous report, 3D^{pol} also replicated RNA in a primer-dependent manner and a self-primed (small stem-loop) manner. Moreover, the initiation of replication was severely dependent on the 3'-terminal structure of RNA, and especially, 3D^{pol} preferred the very small self-primed structure than the single- or double-stranded structure. And the initiation form may be dependent on the divalent ions (Mg²⁺ or Mn²⁺). From these results, we were able to draw a scheme of the *in vitro* primer-independent RNA replication by 3D^{pol}.

Using the IVV displaying 3D^{pol}, we designed the one-pot isothermal replication reaction system, in which the IVV can amplify itself, and evolve autonomously. This reaction system will evolve not only the RNA replicase but also the amplification algorithm itself.

2PT147 *In vitro* selection of a protease using cDNA display

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The artificial evolution of enzymes is one of the ultimate goals of evolutionary molecular engineering. *In vitro* display technologies, such as phage display, ribosome display, mRNA display and cDNA display have been widely used for affinity selection against various targets in the last two decades¹. Conversely, although some groups have been able to evolve enzymes using phage display², there are only a few examples describing mRNA display³ and ribosome display⁴.

Recently, we have started the challenge to evolve enzymes *in vitro* using cDNA display, which is more stable and easy-to-use than mRNA display⁵. We designed a selection system with a new puromycin linker of cDNA display for *in vitro* selection of a protease. The linker was designed to release the cDNA coding the gene of a displayed protease, from a solid-phase support when a peptide substrate was cleaved by the displayed protease.

In this study, we demonstrated a model selection of subtilisin, one of well identified protease. We will discuss the model screening experiments and present some results.

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2PT148 ヒトレチノイン酸結合蛋白質をスカフォールドとする蛋白質のスクリーニング

Screening of mutant proteins scaffolding human cellular retinoic acid binding protein 2

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Proteins have ability to bind other molecule specifically. The creation of artificial specificity of proteins has been studied. The proteins developed by these studies are expected to be applied to pharmaceuticals or drug carriers. Our objective is to create the library mutant human retinoic acid binding protein 2 (HCRABP2), and to screen novel binding ability to vascular endothelial growth factor (VEGF). Sixteen amino acid mutations were introduced into HCRABP2 at the side surface of beta-barrel. Phage display library of the mutants were constructed. Display phage clones were selected through three rounds of affinity panning. The affinity of concentrated display phages was analyzed; and the positive signals were obtained by ELISA analysis. This result suggests existence of clones which might bind to VEGF. Detailed analysis of VEGF-binding affinity and sequence of mutant clones is in progress.

2PT149 マイクロ波照射下での PCR 反応の DNA ポリメラーゼの特异性

The DNA Polymerase Specificity on Microwave Assisted PCR

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Microwave has shown its various features in various chemical reactions, the time shortening, the reduction of reaction temperature, and so on. The microwave effect can be also applied to biocatalysts and enzyme reactions. We have studied to take advantage of the microwave heating to the field of chemical biology and biotechnology. Among them, the polymerase chain reaction (PCR) for gene amplification under microwave irradiation was obtained as a unique result. In PCR method, the three processes are repeated, (1) the thermal denaturation to the single-stranded DNA from the double strand DNA, (2) the hybridization by annealing between the primer and the single-strand DNA, (3) the enzymatic DNA elongation from the primer by DNA polymerase. So that, we demonstrated the microwave heating for the processes of the thermal denaturation and the enzymatic elongation reaction. The Vent DNA polymerase and ExTaq DNA polymerase were used in this study. The ExTaq polymerase was decreased the fluorescence intensity by the microwave irradiation. On the other hand, the Vent polymerase was shown same intensity of fluorescence between the microwave irradiation and the conventional condition. The ExTaq polymerase showed the different phenomena between the microwave irradiation and the conventional heating in spite of same temperature. It is seems that the protein structure is changed under the microwave irradiation. This fact might mean a new state of protein denaturation, what we would call "Microwave Denaturation".

2PT150 モルテングロビュール状態の蛋白質を利用した新規抗腫瘍複合体の作製

Development of novel anti-tumor complexes made from a protein in the molten globule state

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Human α -lactalbumin (α -LA)-oleic acid complex, named HAMLET (human α -lactalbumin made lethal to tumor cells), induces apoptosis-like death of tumor cells, and α -LA in the HAMLET is in a molten globule (MG)-like conformation. Our previous studies have suggested that α -LA in the anti-tumor complex may act as a carrier and that oleic acid in the complex may act as an apoptosis inducer in tumor cells. We have thus assumed that α -LA in the MG state can be used as a delivery carrier of anti-cancer drugs to tumor cells. To investigate this possibility, first, we attempted to make complexes between α -LA and various anti-cancer drugs. As a results, certain anti-cancer drugs form a complex with α -LA in the MG state at pH 2.0, and these complexes were stable under physiological conditions (pH 7.4). Next, we tested cell toxicity of these complexes against various cells. Interestingly, these α -LA-anti-cancer drug complexes exhibited stronger toxicity against tumor cells, but weaker toxicity against normal fibroblast cells than the free drugs. This finding demonstrates that α -LA in the MG state may be a potential drug carrier for cancer therapy.

2PT151 計算化学と実験から考察した、黄色ブドウ球菌のヘム鉄結合・輸送機構解析

Computational and experimental studies on heme-binding and heme-transport mechanisms of Isd proteins of *Staphylococcus aureus*