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Title of Doctoral Dissertation

Developing novel methods for protein crystallization
(新規タンパク質結晶化法の開発)

The structure of proteins is essential for understanding the structure-function relationship of proteins which play an important role in biological and medical science, such as elucidating the mechanism of biological phenomena, structure-based drug design, and industrial enzyme engineering, etc. To determine the 3-D atomic structure of proteins, X-ray diffraction or neutron diffraction of crystals is a powerful method. However, the preparation of protein crystals good enough for diffraction experiments is still the main problem because the theory of crystallization is still unclear, resulting in no efficient and practical method for protein crystallization. Generally, the process of crystallization that crystals form from protein solution, can be divided into three steps: (1) the nucleation, (2) crystal growth, and (3) termination. In this study, we focus on the first two steps.

I. Enhancement of protein crystallization through heterogeneous nucleation

Nucleation is classified into homogeneous and heterogeneous cases. Homogeneous nucleation occurs in the solution without preferential nucleation sites. Many approaches have been developed to increase the probability of nucleation, such as high-throughput methods by using the robotic systems, crystallization screening kits, magnetic/electric field, microgravity, laser irradiation, protein polymerization. However, these methods still largely depend on trial and error, and none of them has been proven as an efficient method in X-ray/neutron crystallography.

Unlike homogeneous nucleation, heterogeneous nucleation happened at the surface of nucleants (solid materials). A variety of nucleants such as minerals, synthetic zeolites, horse and human hair, charged surfaces, porous hydrophobic membranes, mesoporous bio-glass, and molecularly imprinted polymers has been reported in the crystallization of some typical proteins. Crystallizing new and difficult proteins by nucleants are seldom reported. So far, the development of efficient and practical nucleants is still underway.

Considering the advantages of homogeneous and heterogeneous nucleation, we plan to develop nucleants for initializing protein molecular packing. To form crystal, molecules should regularly assemble, and then heterogeneous nucleation starts by initializing molecular packing on nucleant surfaces. For this purpose, we chose the lattice-surface of nucleants for initializing molecular packing, i.e. inducing the nucleation of protein crystals. Furthermore, according to experimental results, we found that nucleants with short lattice distance ($< \sim 10 \text{ \AA}$) is unable to promote crystallization of protein molecules because of large volume size ($\sim 30 \text{ \AA}$ to $75 \text{ \AA}$) proteins. Subsequently, we selected and designed novel nucleants with large

lattice distance as possible as we can synthesize. As a result, we found that Co-cage-1, a metal coordination framework with 37.5 Å lattice distance, facilitates the nucleation of the protein crystal. Among 13 tested proteins, nine of which are very difficult to crystallize and have been tried for long time, successful crystallization (six crystal structures have been determined) of 11 proteins (11kDa~110kDa) proves the capability of Co-cage-1. In addition, we discovered that the surface topography, also plays a key role in nucleation besides the lattice. Furthermore, in situ the observations of nucleation on Co-cage-1 surface are performed by using cryo-transmission electron microscopy (Cryo-TEM) and high-speed atomic force microscopy (HS-AFM). Based on the results, we proposed a heterogeneous nucleation mechanism. The findings of this study open the door to design the nucleants for crystallizing the proteins rationally.

II. Growth of huge protein crystals for neutron protein crystallography

Neutron macromolecular crystallography (NMC) is a powerful method to obtain the accurate position of key hydrogen atoms in protein structures, which are difficult to visualize by X-ray crystallography lonely. However, among 133,500 crystal structures deposited in Protein Data Bank, only about 120 structures (MW < 24 kDa) are determined by neutron diffraction. One of the challenges in NMC is to crystallize the crystal of large volume ($> 1 \text{ mm}^3$), specifically for large protein (MW > 60 kDa) with large unit cell ($> 150 \text{ Å}$).

Our target protein is glutamine amidotransferase CAB (GatCAB), in which the ammonia is proposed to transfer through the inner channel of 30 Å in length. To visualize the ammonia in the channel, neutron diffraction studies are indispensable. However, the following three reasons make GatCAB a difficult sample for neutron diffraction: (1) the largest parameter of unit cell $c = 180 \text{ Å}$; (2) MW is 110 kDa; (3) needle sharp of GatCAB crystal is difficult to grow large volume crystals. Various methods failed to grow large GatCAB crystals, such as macro-seeding, double seeding, agarose gel, and surface mutation.

Inspired by the previous study that the crystal growth rate can be significantly slowed down or accelerated by rearranging structured water molecules around both the surfaces of the protein molecules and the crystal, we adopted the temperature-responsive copolymer (TRCP) to adjust the crystal growth at its lower critical solution temperature (20 °C). As a result, we successfully obtained the large GatCAB crystals up to a volume of $\sim 1.8 \text{ mm}^3$. We collected neutron diffraction data and solved the neutron structure. These results represent a challenge in current neutron diffraction technology.