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Structural Studies of the Protein-Protein Interactions Involved in Vesicular Transport and Autophagy by Synchrotron X-ray Crystallography.

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Introduction

Eukaryotic cells have evolved an elaborate membrane system that allows them uptake of macromolecules from cell exterior, secretion of biosynthetic molecules and metabolism of their component. Membrane traffic is responsible for transport of biomolecules between the membrane-enclosed compartments such as the endoplasmic reticulum, the Golgi apparatus, endosomes, lysosomes and auto-phagosomes. The membrane traffic is organized and regulated by a number of proteins. To understand the mechanism of membrane traffic, it is necessary to know how proteins interact with each other. X-ray crystallography is one of the most powerful techniques to study protein-protein interactions because it provides us with atomic pictures of complexes. Using this method as well other biochemical analyses, my thesis project focuses on structural studies of such interactions in two cases: (1) γ1-ear domain and accessory proteins, and (2) Beclin and hVps34/p150 complex. Furthermore, in anticipation of single particle structural analysis using next generation synchrotron sources, I carried out a computer simulation to examine how residual water molecules affect the diffraction intensities from biological molecules.

Interaction between γ1-ear domain and accessory proteins

γ1-Adaptin is one of the subunits of a heterotetrameric complex AP-1, which is involved in vesicular trafficking between the trans-Golgi network (TGN) and endosomes. Its C-terminal ear domain (γ1-ear) modulates the function of the clathrin-coated vesicles through interactions with accessory proteins such as γ-synergin, rabaptin-5 and EpsinR. γ1-ear also interacts with GGA1, another adaptor protein involved in the vesicular trafficking between TGN and endosomes. To characterize these regulatory interactions, I carried out biochemical and crystallographic analyses of γ1-ear/peptide complexes.

Surface plasmon resonance (SPR) showed that peptides from the accessory proteins and GGA1 have affinities on the order of 10^{-6} – 10^{-4} M. Crystal structures of γ1-ear in complex with peptides show that the conserved basic residues create two hydrophobic cavities, which recognize two aromatic residues in amino acid sequences FGEF and WNSF derived from γ-synergin and GGA1, respectively. Each peptide has an extended conformation in the complex and three residues C-terminal to the second phenylalanine bind a hydrophobic patch to stabilize the protein-peptide interaction (Fig.1). Based on the complex structures and further SPR measurements of mutated peptides, I conclude that [F/W]xxΦ is the determinant for the γ1-ear

binding sequence and, in addition, non-conserved adjacent residues raise the affinity.

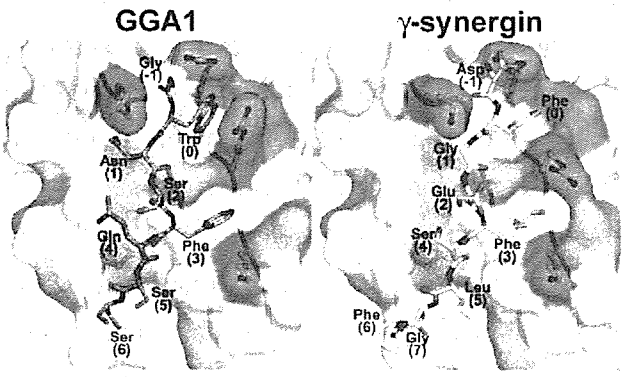

Fig.1 Conformations of two peptides and molecular surface of γ1-ear in the complex
Semi transparent molecular surfaces are colored red, purple and green for acidic, basic and hydrophobic side chains, respectively, and are superimposed with the final models of the complex.

Interaction between Beclin1 and hVps34/ p150 complex

Autophagy is an intracellular degradation system for the majority of proteins and some organelles. The genetic analysis in yeast identified 16 genes (atg1-16) required for autophagy and most of them are conserved in mammals. Beclin1 is a human homologue of the yeast Atg6p and interacts with hVps34/p150 complex, which produces a phosphatidyl- inositol 3-phosphate required in the initial step of autophagy. Beclin1 can also interact with Bcl-2, an important regulator of apoptosis. Thus crystallographic studies of Beclin1 are expected to shed light not only on the mechanism of autophagy but also on the relationship between autophagy and apoptosis.

The first attempt was to express, purify and crystallize the full length of Beclin1 (residue 1-450). However, a heterogeneous aggregation in solution hampered the crystallization trials. To obtain samples suitable for crystallization, I made five deletion mutants according to secondary structure prediction based on its amino acid sequence, and evaluated expression and purification of each mutant. As results, Bec□5 (residue 269-450) and BecΔ7 (residues 142-450) could be purified to crystallization quality. Although the solubility of BecΔ5 was low (~0.4 mg/ml) for crystallization trials, the solubility was increased up to 5 mg/ml when two cystines (residues 353 and 391) were mutated to serines. The initial screening of crystallization conditions gave crystals, and the refinements of the crystallization condition were carried out. However, the crystals were still too small, and did not give any diffraction spots (Fig.2).

Although crystals of Beclin1 could not be obtained, some interesting data about Beclin1 molecule were obtained such as oligomerization mediated by the coiled-coil region and importance of Cys375 in the folding of the Beclin1 C-terminus domain.

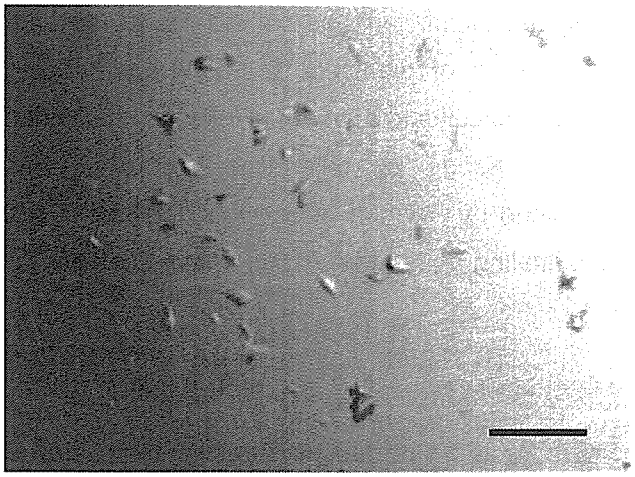

Fig.2 Crystals of BecΔ5
The bar in the picture represents a length of 0.1 mm.

Effect of water molecules upon the diffraction intensities from biological macromolecules

One of the most important challenges in structural biology is to perform structural analysis of biological macromolecules without

crystallization, because many of biologically important targets, such as membrane proteins, multi-domain proteins and supramolecular assemblies, are difficult or impossible to crystallize. Short, intense and coherent X-ray pulses from the forthcoming X-ray sources such as X-FEL and ERL has a possibility to record diffraction data from non-crystalline samples. While the oversampling method is expected to provide solution for the problem of direct phase retrieval, hundreds of thousands of diffraction patterns need to be assembled from identical copies of macromolecule in order to obtain a three dimensional structure at atomic resolution. Radiation damage will be a severe problem since even one intense X-ray pulse of X-FEL will destroy a single macromolecule. Hence, reproducibility of single particles will be of crucial importance. Many factors can affect the reproducibility of samples causing heterogeneity, and a water molecule is one of them. Therefore, computer simulation was carried out to evaluate the change of diffraction intensities by adding water molecules to a model of a protein molecule.

The change of diffraction intensities was examined by *R*-factor, which is extensively used in crystallography. Here *R*-factor is defined as

$$R(I) = \frac{\sum_{hkl} \left| I_{pw}(hkl) - I_{p}(hkl) \right|}{\sum_{hkl} I_{pw}(hkl)}$$

where I_{pw} and I_p represent diffraction intensities of the model containing a protein and water molecules and those of the model containing only the protein molecule, respectively. A certain number of water molecules were selected randomly from a solvent model, added to a protein model, and the diffraction intensities were calculated. The crystal structure of γ1-ear/γ-synergin peptide complex was used as the model of protein and solvent. Averaging of diffraction images were also carried out. Fig. 3 shows *R*-factors of un-averaged and averaged diffraction images as a function of number of water molecules added to the model. If we use 0.166 as the threshold of *R*-factor based on the experiment in Miao *et al.* PNAS (2001), *R*-factor exceeds this at 2 % of water molecules in the case of non-averaging. On the other hand, averaging of diffraction images decreases the *R*-factor significantly and the acceptable level of water molecules increases to 7 % using the same *R*-factor threshold, 0.166. From these results, it is concluded that (1) averaging can decrease the difference between diffraction images with and without water molecules, and (2) the heterogeneity corresponding to 7 % of electrons of a macromolecule can be acceptable for structure determination from diffraction images of single molecules.

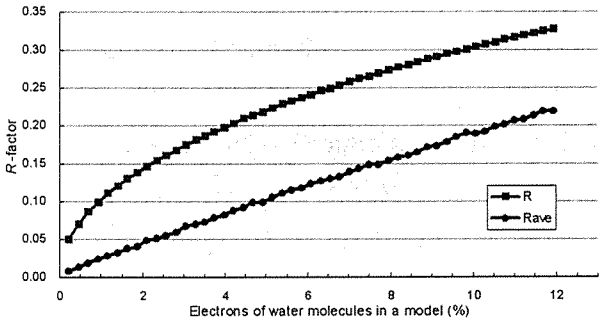

Fig. 3 *R*-factors versus the number of water molecules. Dark blue and yellow lines represent the *R*-factors of un-averaged and averaged diffraction images, respectively. Number of water molecules is represented by the ratio of the number of electrons to a protein molecule. Miao *et al.* have carried out reconstructions successfully using oversampling method with diffraction images to which Poisson noise was added at the level of *R*-factor of 0.166.

Summary

In this thesis, the transient interaction between γ1-ear and accessory proteins were observed and

characterized owing to the successful crystallization of peptide tagged protein. On the other hand, the interaction between Beclin1 and hVps34/p150 complex could not be elucidated by crystallography because of the failure in purification and crystallization of Beclin1. Possibilities of a successful structural analysis of Baclin1 were suggested from the experience in this thesis. However, in order to understand more generally protein-protein interaction occurring in living cells, more structural analyses are needed and it is necessary to develop a method which can be applicable to more varieties of samples. Single molecule imaging is the case with this. A further simulation and investigation will consolidate this method and open the way to a new future of structural biology.

論文の審査結果の要旨

真核細胞内には生体膜によって囲まれた細胞小器官が存在し、それらの間で物質のやり取り(膜輸送)が行われることで、細胞の高度な生命活動が維持されている。この膜輸送の異常が原因となる病気も見つかっており、この細胞内膜輸送を理解することは、細胞生物学のみならず、医学の観点からも非常に重要である。本論文では、細胞内膜輸送の中心に位置するトランスゴルジ網に着目し、そこで物質輸送を担うクラスリン小胞の形成制御にかかわる AP-1 複合体とアクセサリータンパク質との間の相互作用および自食作用に関与する Beclin1 と Vps34/p150 との間の相互作用について、結晶構造解析を用いて研究を行った。また、これらの実験を通して問題となった結晶化の問題を解決すべく、結晶を必要としない単分子の構造解析法について、コンピュータシミュレーションを行い、考察した。

(1) γ1-ear ドメインとアクセサリータンパク質の相互作用

γ1-ear ドメインはクラスリン被覆小胞の形成を制御する AP-1 複合体の一部であり、クラスリン被覆小胞にアクセサリータンパク質と呼ばれる、いくつかのタンパク質を取り込む働きを持っている。そこで、γ1-ear はどのようにアクセサリータンパク質を認識しているのかを調べるためにそれら複合体の構造解析を行った。

これまで、γ1-ear の結合モチーフとして[D/E] FXXF という配列が提唱されていたが、γ1-ear と GGA1 との間で生化学的な結合実験を行い、GGA1 のヒンジ領域にある WNSF というそれまでに報告のなかった配列と結合することを明らかにした。さらに、この WNSF ペプチド、及び[D/E] FXXF モチーフに相当する FGEF ペプチドをN末端に付加することでγ1-ear との複合体結晶構造解析の分解能を飛躍的に改良するという方法を開発し、γ1-ear がより一般的な[F/W]xxΦを結合モチーフとして認識しているという新たな知見を見いだした。

(2) Beclin1 と hVps34/p150 の相互作用

Beclin1 は hVps34/p150 複合体と相互作用して、自食作用(オートファジー)を進行するのに必須なタンパク質である。この分子機構を明らかにする第一段階として、Beclin1 単体の結晶構造解析に向けたタンパク質の発現、精製、結晶化を行った。その結果、断片化タンパク質で、システインをセリンに置換する変異を導入することで、微小ながらも、初めて結晶を得ることに成功した。

(3) 単分子構造解析の際にタンパク質分子表面に存在する水分子の許容量に関する考察

(1)(2)の実験を通して、結晶化が構造解析の大きなボトルネックとなっていること

が、改めて示された。現在、このように結晶化が困難な複合体の構造解析の場合、次世代の放射光源を用いることで結晶でなくとも構造解析の行える可能性のある単分子構造解析が提案されているが、その実現に向けて単分子解析の際に問題となる水の不均一性についてコンピュータシミュレーションを行った。水分子を付加させていないタンパク質モデルと水分子の付加したタンパク質モデルで、回折パターンを計算しその違いをR値で評価したところ、電子の数にして2.7%の水まで付加させても、解析可能な回折データを得られるという結果が得られた。さらに平均化を行うことで付加させられる水分子の数は増大し、8.7 %まで付加可能となることが示唆された。これらの結果は、単分子構造解析を実際に行うにあたって、水分子が付加した状態でも十分に構造解析が可能であるという数学的な可能性を明らかにしたものである。

以上の研究から、タンパク質の細胞内輸送に関わる重要なタンパク質についての放射光X線を用いた構造解析からGGAタンパク質の相互作用に関する顕著な知見を得、次世代放射光X線を用いた構造解析における水分子の重要性について有用な示唆を与えるなど構造生物学研究において有意義な成果を得ることができた。