Graduate School of Science and Technology Doctoral Thesis Abstract

Laboratory name (Supervisor)	Functional Supramolecular Chemistry Laboratory (Prof. Shun Hirota)		
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Title	Studies on construction and stabilization of hemoprotein supramolecules with helical linkers ヘリカルリンカーを用いたヘムタンパク質超分子の構築と安定化に関する研究		

Chapter 1. General introduction

Supramolecular proteins are assembled by protein molecules through noncovalent interactions. They are useful for developing new functional biomaterials, which exhibit unique properties that are not present in single molecules. Protein oligomers can be produced by three-dimensional domain swapping (3D-DS), where identical domains are exchanged between protein molecules through the hinge region. Our group has focused on hemoprotein oligomerization through 3D-DS. However, the arrangement of proteins can easily change in 3D-DS by the loop formation at the hinge region. In this study, a helical linker is used at the hinge region to improve the arrangement of hemoproteins for two cases: 1) Stabilization of a building block protein based on the *Aquifex aeolicus* (AA) cytochrome (cyt) c_{555} trimer, and 2) investigation of the relationship between 3D-DS and heme insertion in myoglobin (Mb) (Figure 1).

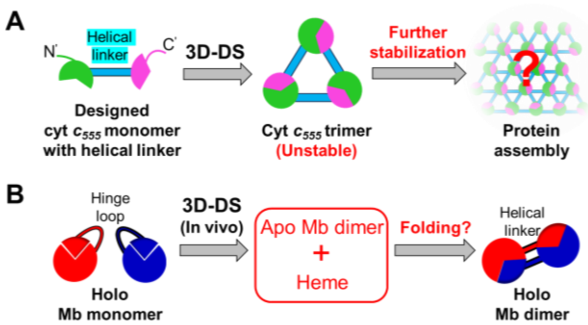

Figure 1. Schematic representation of the present study: (A) Stabilization of a building block protein based on the cyt c_{555} trimer. (B) Investigation of the relationship between 3D-DS and heme insertion in Mb.

Chapter 2. Construction of a cyclic regular-triangle trimer of helix-linked cytochrome c_{555} using sortase A

Precise arrangement of building blocks is important for controlling the protein assembly. The building block protein (cp-c_{555})$_3$, a 3D-DS regular-triangle trimer, has been constructed from cp-c_{555}, which is an α-helix-linked circular permutant of AA cyt c_{555}. However, the trimers may dissociate to monomers in (cp-c_{555})$_3$. To stabilize the triangle structure, a cyclic regular-triangle of three α-helix-linked cyt c_{555} molecules is constructed by covalently connecting terminal regions using sortase A-mediated ligation (SML). Six variants

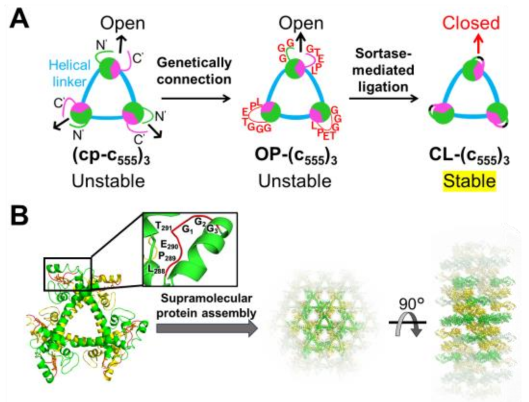

Figure 2. (A) Schematic representation of construction a stable cyclic regular-triangle CL-(c_{555})$_3$ utilizing SML. (B) Crystal structures of CL-(c_{555})$_3$ and its nanoporous supramolecular assembly.

of cp-c_{555} with different N- and C-terminal sequences were subjected to SML, in which the variant with GGG at the N-terminal and LPETG at the C-terminal reacted most efficiently. OP-(c_{555})$_3$, a genetically connected molecule of three α-helix-linked cyt c_{555} molecules containing the optimized sequence for SML, was designed to increase the SML product yield. OP-(c_{555})$_3$ was expressed in *E. coli* cells and the terminal regions were connected by SML, generating a cyclic regular-triangle CL-(c_{555})$_3$ (Figure 2A). CL-(c_{555})$_3$ showed higher thermostability than (cp-c_{555})$_3$ and OP-(c_{555})$_3$. The structural stability of CL-(c_{555})$_3$ was confirmed by high speed-atomic force microscope observation. The crystal structure of CL-(c_{555})$_3$ revealed two stacked CL-(c_{555})$_3$ triangle molecules (Figure 2B) with covalent linkage across the terminal regions (red loops in Figure 2B). Additionally, the stacked CL-(c_{555})$_3$ triangles packed into a nanoporous supramolecular structure (Figure 2B), constructing two pores with diameters of approximately 16 and 30 Å. Stabilization of the building block by cyclization likely facilitates the formation of the nanoporous supramolecular protein assembly in the crystal.

Chapter 3. Apoprotein intermolecular interaction and heme insertion for 3D domain swapping in Mb

Many hemoproteins undergo 3D-DS, yet the relationship between 3D-DS and heme insertion in Mb remains unclear. The crystal structure of the 3D-DS wild-type (WT) Mb dimer revealed the conversion of the hinge loop between E and F helices into a helical structure (Figure 3A). To construct stable 3D-DS Mb dimers, one to three Ala residues were introduced into the hinge region: G80A (K_3AH_2), G80A/H81A (K_3A_2H), and G80A/H81A/H82A (K_3A_3), all of which exhibited the same 3D-DS dimer structure as the 3D-DS WT Mb dimer. Upon expression in *E. coli* cells, the Mb dimer ratio increased in the order WT (1%) < K_3AH_2 (14%) < K_3A_2H (35%) < K_3A_3 (78%), which was consistent with the dimer ratio order obtained by heme reconstitution from the apoprotein. The SEC-MALS analysis confirmed the existence of apo K_3A_3 Mb dimers in addition to monomers. The apo K_3A_3 Mb dimer exhibited larger Cotton effects than its monomer and apo forms of other variants, indicating that the helical linker stabilizes the dimer. Molecular dynamics studies supported the hypothesis that stabilization of the α-helices in the apo K_3A_3 Mb monomer may enhance dimer formation in K_3A_3 Mb compared to WT Mb and other variants. These results suggest that the formation of 3D-DS K_3A_3 Mb dimers in vivo depends on the folding pathway (Figure 3B).

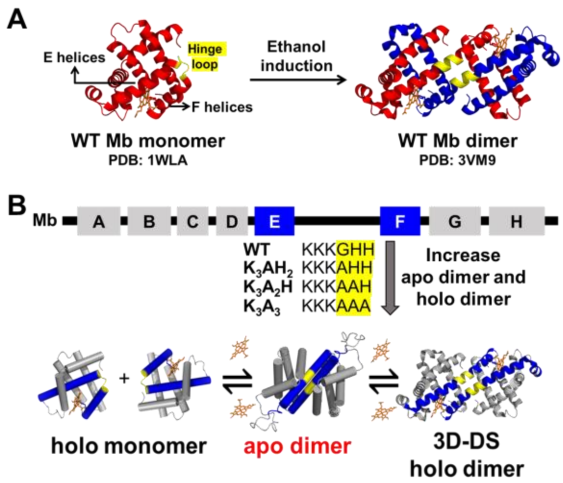

Figure 3. (A) Crystal structure of 3D-DS WT Mb dimer that linked with helical structure, (B) Schematic representation of the helical linker with Ala residues, increasing apo Mb intermolecular interactions and holo Mb dimer formation in vivo.

Chapter 4. Conclusions

Stabilization of hemoproteins is achieved by introducing a helical linker at the hinge region of the 3D-DS structure. The helical linker, together with cyclization by SML, may stabilize the central hole of a building block protein, which is beneficial for the development of nanoporous supramolecular protein assemblies. The conditions for in vivo and in vitro 3D-DS of Mb are clarified. This study shows that helical linkers are useful to increase the stability of a building block hemoprotein as well as to investigate the folding conditions of 3D-DS structure in hemoprotein.

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（論文審査結果の要旨）

タンパク質超分子はバイオマテリアルへの応用が期待され、盛んに研究が行われている。タンパク質超分子を作製する場合、ユニット間を繋ぐリンカー部位が必要となるが、3 次元ドメインスワッピング（3D-DS）のヒンジ領域などのリンカー部位がループ構造を有する場合、超分子構造は不安定なことが多い。そこで本論文では、タンパク質超分子の安定性を向上させるために、剛直なヘリカルリンカーを利用し、ソルターゼ A を用いて空孔を有する安定な環状分子を超好熱菌由来シトクロム（cyt）c_{555} を基に作製するとともに、3D-DS におけるアポ型ミオグロビン（Mb）の分子間相互作用とヘム挿入の関係を調査した。本論文で得られた成果は以下の通りである。

１．空孔を有する安定な正三角形構造の環状タンパク質を作製するため、cyt c_{555} 2 分子を α ヘリックスで融合し、融合タンパク質の N 末端に同じ α ヘリックスを介して cyt c_{555} の C 末端、融合タンパク質の C 末端に同じ α ヘリックスを介して cyt c_{555} の N 末端を融合して OP-(c_{555})$_3$ を作製した。次に、OP-(c_{555})$_3$ の N 末端と C 末端をソルターゼ A を用いてペプチド結合で繋いで CL-(c_{555})$_3$ を得た。N 末端のアミノ酸配列が GGG、C 末端のアミノ酸配列が LPETG の場合、ソルターゼ A の触媒反応が効率的に進むことを明らかにした。CL-(c_{555})$_3$ は環状化により OP-(c_{555})$_3$ より熱安定性が向上した。X 線結晶構造解析により、CL-(c_{555})$_3$ は N 末端と C 末端が共有結合で繋がり、さらに、結晶中で CL-(c_{555})$_3$ が積み重なって直径約 16 Å と 30 Å の 2 つの細孔を有するナノ多孔性超分子構造を構築することを明らかにした。

２．ヒンジ領域にアラニン（Ala）を 1～3 個挿入した Mb の変異体を用いて、アポタンパク質の分子間相互作用に着目し、Mb における生体内での折り畳み中でのアポ型 Mb の 3D-DS 2 量体の安定性とヘム挿入の関係を体系的に調査した。SEC-MALS 分析により、ヒンジ領域に 3 つの Ala 残基が導入された G80A/H81A/H82A（K_3A_3）変異体のアポ型は、単量体に加えて 2 量体を形成することを明らかにした。アポ型 K_3A_3 Mb の 2 量体は、単量体や他の変異体のアポ型よりも多くの 2 次構造を有し、ヘリカルリンカーが 2 量体の安定化に関与していることが示された。さらに、生体内での折り畳み中の 3D-DS K_3A_3 Mb 2 量体形成量がアポ単量体－2 量体の平衡に依存することを提案した。

本論文では、ヘリカルリンカーとソルターゼ A の反応を利用して空孔を有する安定な正三角形構造の環状タンパク質を作製するとともに、Mb の 3D-DS においてヘリカルリンカーを導入し、アポタンパク質の分子間相互作用とヘム挿入の関係を解明した。これらの結果は、タンパク質超分子に新しい知見を与えるものであり、本論文で得られた結果はタンパク質科学分野および生体分子科学分野の研究として高く評価でき、学術的に大きな意義がある。よって、審査委員一同は本論文が博士（理学）の学位論文として価値あるものと認めた。