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学 位 論 文 題 目　State-selective stabilization of adenosine A_{2A} receptor by de novo design of protein structures

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博士論文の要旨

氏　　名 三本 斉也

論文題目 State-selective stabilization of adenosine A_{2A} receptor by de novo design of protein structures

Computational design technology to accurately create protein structures de novo with high thermal stability has greatly advanced in this decade. Based on the technology, I aimed to develop computational methods to stabilize proteins in a selected state.

In this thesis, I targeted on G-protein coupled receptors (GPCRs) for state-selective stabilization. GPCRs are the largest membrane protein family encoded by the human genome. Canonically, GPCRs are activated upon the binding of extracellular ligands, which induces a conformational change from the inactive state to the active state, leading to intracellular coupling of G-proteins that trigger downstream biochemical cascades. Because GPCRs control diverse physiological functions, they have been of major scientific interest and also among the main drug targets. However, despite their scientific and pharmacological importance, the innate instability of GPCRs has been problematic for sample preparation and functional assay. Moreover, GPCRs are in equilibrium between inactive and active states that exhibit large conformational changes upon state transitions. The low homogeneity caused by this feature has been problematic for structure determination and state-specific ligand/antibody screening. In this thesis, I aimed to develop methods that not only stabilize GPCRs but also stabilize them in a specific state.

One of the causes of GPCR instability is the intracellular loop 3 (ICL3), an intracellular loop with varying lengths among different GPCRs that shows high structural flexibility. In addition, ICL3 connects the two transmembrane helices, transmembrane helix 5 (TM5) and transmembrane helix 6 (TM6), which move outward upon the conformational change from the inactive to active state (the agonist-bound G-protein coupled state). I hypothesized that by redesigning the TM5-ICL3-TM6 region to stabilize TM5 and TM6 in either the inactive or active state conformation, state-selective stabilization of GPCRs might be achieved.

I applied two approaches for state-selective stabilization of GPCRs: 1) designing stable TM5-ICL3-TM6 region by redesigning ICL3 to be short and typical structures, and 2) designing extremely stable all α-helical proteins that are made to fix the conformation of TM5 and TM6 by being replaced to ICL3. In this study, I targeted adenosine A_{2A} receptor ($A_{2A}R$) and attempted to stabilize it in either the inactive or active state. $A_{2A}R$ is a prototypical GPCR belonging to class A that constitutes the largest GPCR subfamily covering approximately 90% of human GPCRs.

My thesis consists of five chapters: Chapter 1 provides a brief overview of GPCRs and protein design, Chapter 2 describes the redesign of ICL3 for state-selective stabilization of $A_{2A}R$, Chapter 3 and 4 focus on the de novo design of fusion partner proteins customized to stabilize the inactive and active states of $A_{2A}R$, respectively, and Chapter 5 summarizes the conclusions of the thesis.

In Chapter 2, the computational strategy for redesigning TM5-ICL3-TM6 region and experimental results of the redesigned $A_{2A}Rs$ are described. One reason that can explain the exceptionally high thermal stability of computationally de novo designed proteins is the use of short and typical loop structures. Therefore, I redesigned the TM5-ICL3-TM6 region of the $A_{2A}R$ to have short and typical loop structures. For state-selective stabilization, I redesigned the region of the inactive state structure and active state structure of $A_{2A}R$, respectively. First, I computationally rebuilt ICL3 between TM5 to TM6 as a short and typical structure. Then, sequence design was performed around the cytosolic area of TM5-ICL3-TM6. The redesigned $A_{2A}Rs$ with new ICL3 structures, exhibiting the lowest energy, were selected for experimental characterization. The redesigned $A_{2A}Rs$ were experimentally characterized by Murata group at Chiba University, and the results suggest that the stability of the redesigned $A_{2A}Rs$ was not improved compared to that of the wild-type. This indicate more stabilization for the redesigned region is required.

To improve the method, I computationally de novo designed super-stable protein structures to be replaced to ICL3. In Chapter 3, the computational strategy for the de novo design of extremely stable all α-helical proteins customized for fusion to $A_{2A}R$ and the experimental results are described. The fusion partner strategy, which replaces ICL3 with soluble protein domains (fusion partner proteins), has been widely employed for stabilizing GPCRs. However, this method requires numerous experimental trials and errors. I aimed to develop a computational method to design fusion partner proteins that stabilize GPCRs in a specific state. First, I prepared a set of hundreds of all α-protein backbone models, and selected the backbone models that can be fused into $A_{2A}R$ through straight helical connections. Next, I designed amino acid sequences for the selected backbones. Then, I performed experimental characterizations of the designed proteins. The designed fusion partner protein, named FiX1, folded into a monomeric structure with high thermal stability (the melting temperature is more than 98 °C), and the chimeric $A_{2A}R$ with FiX1 ($A_{2A}R$–FiX1) exhibited higher thermal stability than the wild-type $A_{2A}R$. The ligand-binding affinity of $A_{2A}R$–FiX1 to the inverse agonist was similar to that of the wild-type $A_{2A}R$, while the affinity to the agonist was drastically reduced. These results indicate that the rational stabilization of the $A_{2A}R$ inactive conformational state was successful.

To investigate the potential of the developed method, in Chapter 4, I computationally de novo designed super-stable fusion partner proteins to stabilize $A_{2A}R$ in an active state. The de novo designed fusion partner proteins showed extreme thermostability, and the

chimeric $A_{2A}R$ with a fusion partner protein, $A_{2A}R$–FaX3, exhibited higher stability than the wild-type $A_{2A}R$. However, $A_{2A}R$–FaX3 did not show ligand-binding affinities against the inverse agonist and agonist. These results indicate that $A_{2A}R$–FaX3 was stabilized in an unexpected state other than the inactive or active state. For stabilizing GPCRs in an active state, the TM5 and TM6 region needs to be more stabilized. The experimental characterization of the chimeric $A_{2A}R$s described in Chapters 3 and 4 was performed by Murata group at Chiba University.

I succeeded in stabilizing the inactive state of $A_{2A}R$ by the mainchain-level protein engineering: custom-made design of super-stable fusion partner proteins. The developed method still needs to be improved to stabilize an active state. However, the method is expected to be a basic technology for designing rationally stabilized GPCRs in a desired state, which is thought to be applied in solving structures in specific states or state-dependent ligand/antibody screening. In addition, this is the first example of rational state-selective stabilization of proteins only by mainchain-level engineering, not limited in GPCRs. The method is expected to be utilized to stabilize other proteins in desired states.

Form8・Separate Sheet
（様式８・別紙１）

Results of the doctoral thesis defense

博士論文審査結果

Name in Full
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Title
論文題目　　State-selective stabilization of adenosine A_{2A} receptor by de novo design of protein structures

G タンパク質共役受容体(GPCR)は細胞膜上に存在する受容体の一種であり、細胞外からの多様な信号を受容して構造変化を起こし、細胞質側に結合しているGタンパク質を活性化する。GPCRは細胞外シグナルを細胞内に伝達する役割を担うため、主要な創薬ターゲットとしても注目されてきた。しかしながら、GPCRの熱力学的不安定性や、非活性化状態(信号無し)と活性化状態(信号有り)の動的平衡に起因する不均質性が、構造決定や創薬における障害となっていた。そこで出願者は、計算機および生化学実験を用いて、GPCRの一種であるアデノシン受容体($A_{2A}R$)を状態選択的に安定化する手法の開発を行った。新規設計したタンパク質ドメインを$A_{2A}R$に融合する技術基盤を確立し、$A_{2A}R$の非活性化状態を選択的に安定化することに成功した。

本学位論文は、第一章を序論、第二章から第四章を本論、第五章を結論とする全五章から構成されている。

第一章では、GPCRのシグナル伝達機構、構造、状態特性が示されるとともに、計算機を用いたタンパク質構造の設計手法について詳述されている。

第二章では、$A_{2A}R$の状態選択的な安定化に向けて、その第５および第６膜貫通ヘリックスを非活性化状態または活性化状態に見られる角度で固定する戦略が提案されている。角度を調整する手法として、出願者は、両ヘリックスを連結する細胞内第３ループに着目している。同ループの主鎖を、計算機を用いて自然界のタンパク質に頻出する短いループ構造に改変することで、$A_{2A}R$の状態選択的安定化を図っている。改変型$A_{2A}R$の可溶化効率が野生型$A_{2A}R$よりも低化するという予想に反した結果を受けて、その要因や開発した手法の問題点について考察がなされている。

第三章では、第二章の結果を踏まえ、細胞内第３ループ領域に新規タンパク質ドメインを設計して融合する手法が詳述されている。融合タンパク質と$A_{2A}R$を連結する部位が折り曲がりやループを含まない剛直なαヘリックスを形成し、第５および第６膜貫通ヘリックスが狙った角度で固定されるようテーラーメイドに設計することで、$A_{2A}R$の非活性化状態を選択的に安定化することに成功している。

第四章では、第三章で開発した手法を用いて、$A_{2A}R$の活性化状態を選択的に安定化することに挑戦している。しかしながら、改変型$A_{2A}R$は活性化状態、非活性化状態のいずれにも分類されない別の状態に安定化されるという結果となった。これらの結果を受けて、第三章で開発された手法の問題点や改良方法について考察がなされている。

第五章では、本学位論文を総括し、本研究の将来展望について議論している。

以上のように、本学位論文では、従来困難であったGPCRの状態選択的安定化を達成するための基本設計原理を確立し、生化学実験等をとおして、$A_{2A}R$の非活性化状態が選択的に安定化されることを実証した。$A_{2A}R$がGPCRファミリーの約90%を占めるclass Aに属していることからも、出願者の開発した手法は他のGPCRへの適用性が高く、当該分野の研究の発展に大きく寄与するものと期待される。また、本論文の成果の一部は、既に一報の査読付き国際学術誌に発表されており、国際的にも高い水準の研究であると判定できる。

以上により、本論文は博士（理学）の学位授与に値すると審査員全員一致で判断した。