様式３

論　文　内　容　の　要　旨

氏　名　（　Xin Zhang　）	
論文題名	Solid-State NMR Methodologies for Membrane Proteins and Application to a Halorhodopsin (膜蛋白質の固体NMR解析の方法論とハロロドプシンへの展開)

論文内容の要旨

Membrane proteins, which account for 20–30% of genomically encoded proteomes, are fundamental to a variety of important life activities. Despite their important roles in life science and extensive research interest, understanding of membrane proteins' structure-function relationship remains insufficient, as evidenced by their underrepresentation in the structural database. For example, less than 2% of the protein structures deposited in the Protein Data Bank (PDB) are membrane proteins. The difficulty of structural study for membrane proteins stems from their physicochemical properties, particularly their integration within lipid bilayers. Solid-state NMR with magic-angle spinning (MAS ssNMR) emerges as a promising alternative technique for the structural analysis of membrane proteins assembled in lipid bilayers at atomic resolution under near-physiological conditions. Although MAS ssNMR offers unprecedented access to structural and dynamic information, it is still a new method and faces challenges related to sensitivity and resolution, necessitating advanced sensitivity enhancement strategies and new sample preparation protocols.

In this study, I worked on the structural analysis of *Natronomonas pharaonis* halorhodopsin (*Np*HR) an archetypical Cl^- ion inward pump derived from thermophilic archaea. *Np*HR inwardly pumps Cl^- into cells for regulating osmotic pressure with light irradiation and has been widely used as an optogenetic tool. Natively, *Np*HR forms a homotrimer in cell membrane and each monomer contains seven transmembrane helices (namely helix A-G) with a retinal chromophore binding to the sidechain of the Lys256 residue through Schiff base.

The light driven Cl^- transport function of *Np*HR is coupled with a cyclic conformational change which is called photocycle. The photocycle is generated by the light-induced isomerization of the retinal, leading to a series of structural changes in the whole protein that facilitate Cl^- transport and ended by the restoration of the ground state. Despite extensive X-ray crystallographic studies having been performed and revealed the molecular details of the ion release step in the early photocycle, the Cl^- uptake mechanism involved in the late photocycle remains unclear.

The mechanistic uncertainty becomes apparent when examining the crystal structures of *Np*HR both in the ground Cl^--bound state and the Cl^--free state, the latter representing an established mimic of the photocycle's O intermediate. Both structures display a similar closed conformation, contradicting the anticipated open conformation preceding the Cl^- uptake. Moreover, the extracellular side of this O-mimic state is entirely obstructed by the large extracellular loop region (B-C loop), making it difficult to identify any Cl^- uptake pathway. Crystal packing, which restricts large molecular motion, may contribute to the observed limited structural differences. This situation highlights the uniqueness of ssNMR analysis under near physiological lipid bilayer conditions for elucidating the Cl^- uptake process in the late photocycle in debate.

In the first part of this study (Section 2). I first developed a refined unfolding and refolding protocol for preparing the perdeuterated *Np*HR with enhanced amide proton (^1H_N) back exchange in its native trimeric state for ^1H detection ssNMR. While this perdeuteration and ^1H_N back exchange step proved essential for obtaining high resolution ^1H detection ssNMR data, achieving sufficient ^1H_N back exchange

represents a challenge in membrane proteins due to limited solvent accessibility, resulting in severe signal losses. My protocol, although retaining some signal losses, significantly improved the $^{1}H_{N}$ back exchange efficiency. This enabled me to obtain 3D CANH spectra with enhanced information content using approximately 1 mg sample. I observed numerous peak shifts in the spectra between the Cl^--bound and -free states, which indicated significant structural modifications between the two states widely distributing in the molecule.

In the second part of this study (Section 3), I performed signal assignments for *Np*HR using ^{13}C detection ssNMR and successfully assigned residues in major part of helix D, the C-D and E-F loop and some residues in other helices. Through chemical shift perturbation (CSP) analysis, which reflects local environment changes, I have revealed that one of the significant structural changes was located at the extracellular (EC) part of helix D between the Cl^--bound and -free states.

Based on the above data, I examined in the final part of this study (Section 4) the functional implications on the identified structural change to the Cl^- uptake process through targeted mutations. I prepared point mutants of *Np*HR for residues in the EC part of Helix D and analyzed their molecular properties. Notably, the Ala to Val mutant at position 165 (A165V) exhibited distinct Cl^- uptake characteristics, including lower Cl^- binding affinity and a significantly accelerated late photocycle kinetics compared to the wild type. This mutation induced trimer dissociation both in the Cl^--free state and following photocycle activation. These results suggest that an open conformation, which pushes adjacent monomers apart, exists in the Cl^--free state or during the O intermediate. DNP-enhanced ^{15}N detection ssNMR measurement confirmed that the A165V mutant retains protein folding through preserved Schiff base ^{15}N chemical shift, supporting a model where the EC part of helix D undergoes outward movement during O intermediate formation. This movement may be critical for the Cl^- uptake process, facilitating the opening of the Cl^- entrance on the extracellular side, which was not observed in X-ray structures.

This study significantly advances understanding of the Cl^- uptake mechanism of *Np*HR in the late photocycle, while demonstrating the utility of ssNMR for examining structural changes of membrane proteins under near-physiological lipid bilayer conditions.

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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

古細菌 *Natronomonas Pharaonis* 由来ハロロドプシン(*Np*HR)は、細胞の浸透圧バランスを制御する 7 回膜貫通ヘリックスを持つ塩素イオンポンプである。緑黄色光による励起で、蛋白質内に結合したレチナール分子が異性化し、それに伴う一連の蛋白質構造変化（フォトサイクル）を通じて塩素イオンを輸送するが、サイクル後半におけるイオン取り込み過程についての構造的知見が長らく希薄であった。イオン取り込みの前状態（O 中間体）は一種の開状態を取ると考えられるが、結晶構造研究では基底構造（閉状態）と大きな違いが検出されず、議論がつづいていた。

このような状況を踏まえ、本研究では *Np*HR を結晶化することなく、脂質二重膜中で直接解析することに主眼をおき、主に固体 NMR 法を用いた手法開発と応用研究を展開している。その結果、以下の２つの重要な成果を得ている。

まず、これまで明らかになっていなかった O 中間体における *Np*HR の構造変化について具体的な知見を得た。微量（~1 mg）*Np*HR 試料に ^{1}H 直接観測 NMR 法を適用し、基底状態 *Np*HR とその脱塩で得られる擬 O 中間体との間に広範な構造変化が存在することをつきとめた。ここでは情報量を最大化するために *Np*HR 分子の完全重水素化とアミド ^{1}H 原子の再配置を行う新しい試料作製プロトコルも開発した。これに次ぐ ^{13}C 観測多次元 NMR 法(~10 mg *Np*HR 試料)による詳細な解析からは、変化部位はヘリックス D の細胞外側部位であることを特定した。

次に、特定された構造変化部位に複数の変異体を作成し過渡吸収分光、円二色性分光、分析クロマトグラフィーとスピン超偏極固体 NMR データ、さらに既知の X 線結晶構造からの知見を組み合わせ、構造変化と機能の関係を総合的に検証した。この結果、ヘリックス D 細胞外側部位は、生理的３量体中のモノマー界面において外向きに動くと結論した。これまで知られていなかったこの構造変化は、*Np*HR の細胞外表面を被っている B-C ループを避けイオン取り込み経路となる開口部を提供するもので、フォトサイクル後半のイオン取り込み過程に必須と考えられる。

以上の研究成果は、イオンポンプ膜蛋白質の作動機序について、新しい知見を得てその分子機序の理解を拡張するものであり、本論文は博士（理学）の学位論文として十分価値あるものと認める。