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Natronomonas pharaonis halorhodopsin Ser81 plays a role in maintaining chloride ions near the Schiff base

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Optogenetic technologies have often been used as tools for neuronal activation or silencing by light. *Natronomonas pharaonis* halorhodopsin (NpHR) is a light-driven chloride ion pump. Upon light absorption, a chloride ion passes through the cell membrane, which is accompanied by the temporary binding of a chloride ion with Thr126 at binding site-1 (BS1) near the protonated Schiff base in NpHR. However, the mechanism of stabilization of the binding state between a chloride ion and BS1 has not been investigated. Therefore, to identify a key component of the chloride ion transport pathway as well as to acquire dynamic information about the chloride ion-BS1 binding state, we performed a rough analysis of the chloride ion pathway shape followed by molecular dynamics (MD) simulations for both wild-type and mutant NpHR structures. The MD simulations showed that the hydrogen bond between Thr126 and the chloride ion was retained in the wild-type protein, while the chloride ion could not be retained at and tended to leave BS1 in the S81A mutant. We found that the direction of the Thr126 side chain was fixed by a hydroxyl group of Ser81 through a hydrogen bond and that Thr126 bound to a chloride ion in the wild-type protein, while this interaction was lost in the S81A mutant, resulting in rotation of the Thr126 side chain and reduction in the interaction between Thr126 and a chloride ion. To confirm the role of S81, patch clamp recordings were performed using cells expressing NpHR S81A mutant protein. Considered together with the results that the NpHR S81A-expressing cells did not undergo hyperpolarization under light stimulation, our results indicate that Ser81 plays a key role in chloride migration. Our findings might be relevant to ongoing clinical trials using optogenetic gene therapy in blind patients.

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1. Introduction

Halorhodopsin (HR) is a light-driven pump that transfers chloride ions from the extracellular to the cytoplasmic side of the archaeal plasma membrane [1]. It is currently used to control the membrane potential of neurons in optogenetic applications [2]. In the field of vision research, the light-sensitive, cation-selective ion channel ChR2 has been used for restoring vision by an adeno associated virus vector-mediated gene therapy [3,4]. Although optogenetic gene therapy to restore vision remains in the clinical trial stages, it is well-known that in the visual system, center-surround antagonism is a fundamental aspect of visual

Abbreviations: NpHR, *Natronomonas pharaonis* halorhodopsin; BS1, binding site-1; MD, molecular dynamics; HR, halorhodopsin; sHR, *Halobacterium salinarium* halorhodopsin; POPC, phosphatidylcholine; RMSD, root-mean-square deviation; IRES, internal ribosome entry site.

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information processing [5]. Greenberg et al. first demonstrated the center-surround system, using the two optogenetic moderators ChR2 and eNpHR [6]. Similarly, the light-sensitive chloride ion pump of NpHR has an important role in neural circuit regulation.

There are many studies on HR-mediated chloride ion transport that have used HR from *Natronomonas pharaonis* (NpHR) or *Halobacterium salinarium* (sHR). Both HRs possess seven transmembrane helical structures (helix A to G) and have a retinal that is covalently bound via a Schiff base to Lys 256, which is located at the center of the G helix. (Numbers of all amino acids and water molecules used in this study correspond to those of NpHR PDB ID: 3A7K [7].) The absorption of light by the retinal chromophore causes a reaction cycle in which the protein passes through a sequence of intermediate states: HR → K → L1 → L2 → N → O → HR' [8–10]. The last two states are involved in chloride ion release [9] and uptake [11], respectively. The movement of chloride ions through the pump is thought to occur in four distinct steps. In the resting state (all-trans HR) and the first two 13-cis states (K and L1), the chloride ion is located at binding site-1 (BS1) of the coordinate position of Thr126, Ser130, and the protonated Schiff base [12–14]. On the cytoplasmic side, a chloride ion binds to Lys215 and Thr218 on helix F [15] in the L2 state. Structural information on the N and O intermediates is not fully understood, as the O state is not detected in sHR [8]; however, the O-type crystal structure has been obtained in NpHR [16]. This structure indicates that the release of chloride ions into the cytoplasm is accompanied by the outward movement of helix F, as observed during the migration of azide anions [17]. After the cytoplasmic channel is opened, almost all chloride-protein interactions are replaced by hydration of water with chloride ions. The movement of helix F to its original position occurs during the decay of O to HR'. A chloride ion then binds to BS1 again, and the repositioning cycle can be resumed [15]. In the resting state crystal structure of NpHR [7], the retinal is fixed by the amino acid group comprised of Trp127, Tyr180, Phe187, Trp222, Tyr225, and Trp229. Schiff bases are protonated, facing extracellularly in the resting state. A chloride ion is fixed to BS1 located in the cavity near the Schiff base. Furthermore, the chloride ion binds to a water molecule (Wat 502) by a hydrogen bond. As mentioned above, there is a complicated network of hydrogen bonds in the cavities of the ion pathway from outside the cell to the Schiff base, which is involved in the conversion of chloride ions from BS1 to the retinal toward the cytoplasmic side [18].

In this study, we attempted a rough analysis of the chloride ion pathway shape and analyzed the dynamics of the protein-chloride ion binding state, using all-atomic molecular dynamics (MD) simulations with wild-type and S81A mutant NpHR to understand how NpHR controls chloride ion movement. We focused on the role of Ser81 near Thr126 for chloride ion transport in halorhodopsin.

2. Material and methods

2.1. Construction of the model structure

The initial structural coordinates of the NpHR protein were determined based on the ground-state crystal structure of wild-type HR derived from *N. pharaonis* (PDB: 3A7K) [7], using homology modeling server swissmodel (https://swissmodel.expasy.org/) and GENBANK: EF474018 [2] as the sequence information. The model structure contains protein trimers, covalently bound retinal chromophores, three carotenoid lipid molecules (bacterioruberin) [7], 144 water molecules, two chloride ions per protein monomer, and intra-trimer spaces with phosphatidylcholine molecule oleoyl-lipid fragments. Sodium ions were added for charge neutrality.

2.2. Energy minimization and MD simulation

The potential energy function was set as follows: the CVFF force field [19] was used for proteins and lipids, while the extensible and systematic force field, ESFF [20], was used for polyene chains (retinal and bacterioruberin) and chloride ions. The following protocol was employed to perform an MD simulation: A simulation system was geometrically optimized by performing a 5000-step conjugate gradient energy minimization. We fixed chloride ions, protein, and bacterioruberin-heavy atoms as well as lipid and water molecules degraded in the crystal structure during geometry optimization. An equilibration calculation was carried out by progressively decreasing position constraints in 200 ps MD simulations, starting from a force constant of 25 kcal/mol·Å to 10 kcal/mol·Å and 1 kcal/mol·Å, respectively. The 1-ns simulation was then

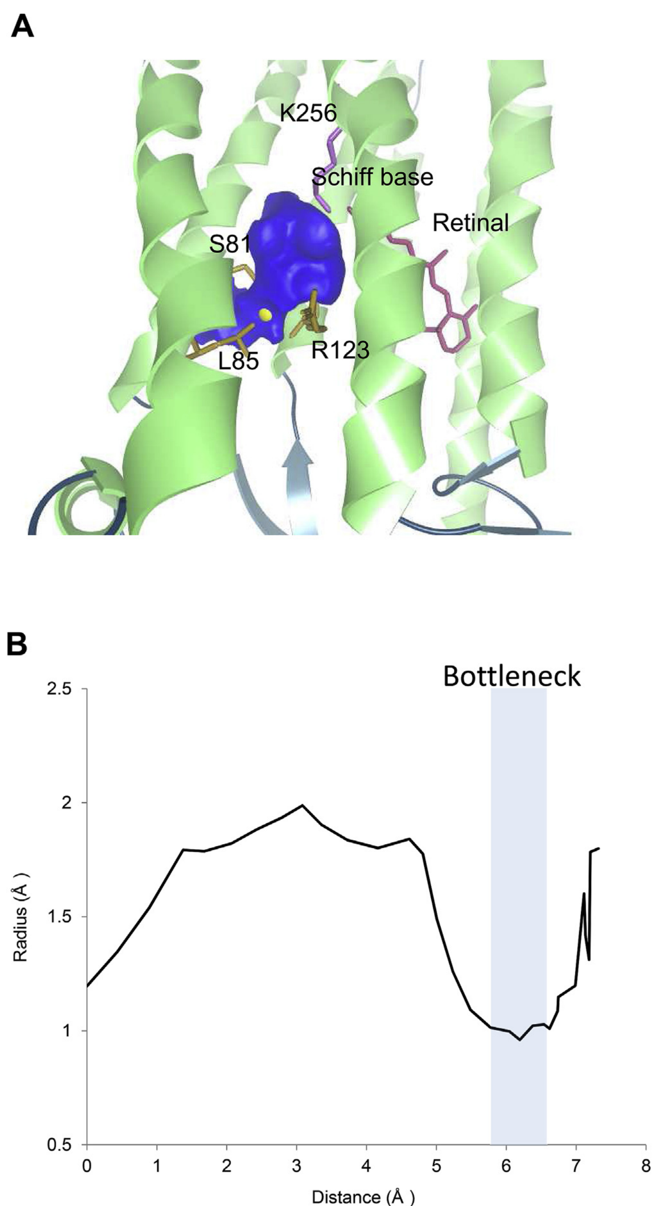

Fig. 1. Detailed shape of the ion tunnel predicted by CAVER near the Schiff base. (A) The blue object surrounded by S81, L85, R123, and Schiff bases indicates the tunnels of predicted ions inside NpHR. (B) The radius of the ion tunnel bottleneck, comprised of S81 and L85, is less than 1.0 Å. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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