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Crystal structure of heliorhodopsin

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Biochemical characterization of *Ta*HeR

We characterized the biochemical properties of *Ta*HeR. *Ta*HeR shares 43% sequence identity with HeR 48C12 and is distant from HeR 48C12 in the phylogenic tree (Extended Data Fig. 1a). However, we observed many similarities between *Ta*HeR and HeR 48C12, as discussed below. *Ta*HeR did not show any ion transport activities in an *E. coli* ion-transport assay with a pH electrode, similar to HeR 48C12 (Extended Data Fig. 1b), and no chloride or sodium ion binding was observed by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) (Extended Data Fig. 1c, d). An HPLC analysis of the extracted retinal revealed that the retinal in *Ta*HeR adopts an all-*trans* configuration in the dark and photo-isomerizes to the 13-*cis* form, as in HeR 48C12 (Extended Data Fig. 1e). *Ta*HeR showed an absorption maximum wavelength (λ_{max}) at 542 nm. The pKa of the retinal Schiff base is 11.2 (Extended Data Fig. 1f) and that of the Schiff base counterion is 3.6 in *Ta*HeR (Extended Data Fig. 1g). These values are comparable to those of HeR 48C12 (λ_{max} = 551 nm, pKa = 11.5 and 3.7 respectively), suggesting that they share similar properties. *Ta*HeR underwent a photocycle involving the K, M, and the long-lived photoactivated O intermediates upon light absorption, similar to that of HeR 48C12 (Extended Data Fig. 2a-d).

Immunofluorescent staining analysis

To further validate its topology, we expressed *Ta*HeR with a c-Myc tag at the N-terminus or C-terminus, and performed an immunofluorescent staining analysis (Extended Data Fig. 3c-e). Only the *Ta*HeR with the c-Myc tag at the C-terminus was immunostained, indicating that the N-terminus faces the cell cytoplasm, consistent with the inverted topology. The current *Ta*HeR structure and functional analysis clearly demonstrate the inverted topology of HeRs (Extended Data Fig. 4f, g).

Mutational study of retinal binding site

To validate the position of the retinal binding site in the crystal structure, we mutated the amino-acid residues present within 4.5 Å of the retinal to alanine (alanine was mutated to serine) (Extended Data Fig. 6b, c). Two mutants showed no absorption change, 11 exhibited a spectral blue shift, 3 exhibited a spectral red shift, and Y109A did not form a pigment, among the 17 mutated residues in total. A previous alanine-scanning

analysis of HeR 48C12, in which the residues distant from the retinal were tested, resulted in ~40% of the variants showing an identical absorption spectrum to the wild-type. The present results show that the residues near the retinal are directly related to the color tuning[1], and the numerous blue-shifts by the alanine substitutions suggest that the residues constituting the retinal binding pocket have a red-shifting effect on the retinal absorption.

Structural dynamics monitored by FTIR

The signaling O state showed a band at 1184 cm^{-1}, and thus the retinal chromophore is presumably in the 13-*cis* form (Extended Data Fig. 7b). The 1009 (-)/999 (+) cm^{-1} bands are considered to be hydrogen-out-of-plane vibrations, implying a distortion in both the dark and O states. The proton transferred to the PAG (hydrogen bonding network including water molecules) in the M-intermediate returns to the Schiff base in the O state, resulting in a larger structural change. The negative band at 1655 cm^{-1} observed in the difference spectra between the M and initial states is shifted in D_2O, and thus it was assigned to the C=N stretching vibration of the Schiff base. In contrast, the 1656 (-) cm^{-1} band observed in the spectra between the O and initial states did not show any deuterium shift, indicating that it represents the amide-I vibration of the helix (Extended Data Fig. 7b). The higher frequency shift to 1694 and 1673 cm^{-1} upon photo-activation suggested the distortion of the helical structure. Similar large amide-I changes of α-helices occur in BR[2], channelrhodopsin-2[3], and animal (GPCR-type) rhodopsins[4], where the lower frequency shifts indicate the strengthened hydrogen bonds upon the opening of TM6. The 1620 (-)-cm^{-1} band suggested the structural changes of the β-sheet in the O intermediate (Extended Data Fig. 7b). As *Ta*HeR only contains β-sheets on the extracellular surface (Fig. 1a), the structural change reaches the extracellular β-sheets in the signaling O intermediate, presumably through the unique helical structural alterations. The accumulation of the O-intermediate decreased in the E227Q mutant, and therefore this Glu227 residue, which is completely conserved in HeRs, is likely to play important roles in these structural changes (Extended Data Fig. 8f).

Comparison of *Ta*HeR and Meta II state rhodopsin

The crystal structure of the Meta II state rhodopsin revealed two openings of the

7-transmembrane (TM) bundle towards the hydrophobic core of the membrane[5]. The computational analysis revealed a putative ligand channel connecting the openings and traversing the binding pocket, which is the putative retinal entrance/exit. However, a previous mutational study did not yield a clear picture which opening is used for retinal entrance and exit, respectively. The lateral fenestration in *Ta*HeR is larger than the two openings in the Meta II state rhodopsin (Extended Data Fig. 9e, f) and our mutagenesis analysis clearly showed that it is the retinal entrance in *Ta*HeR.

Possibility that HeR functions as a photo-activated enzyme.

Typical photoactivated enzymes, bPAC[6] and rhodopsins[7,8], have long-lived photoactive states (τ >1 s). Therefore, the conserved long-lived photoactive states of the HeRs also imply that they function as photoactivated enzymes. The *Ta*HeR structure lacks the hydrophobic pocket observed in other membrane enzyme such as AdipoRs, and thus *Ta*HeR may catalyze a water-soluble substrate.

Another aspect of the type-1 and type-2 rhodopsins is that they have constitutive lipid-scrambling activities. Recently, Dr. Menon's group[9,10] clearly showed that the type-1 and type-2 rhodopsins both possess the scramblase activity, as revealed by a fluorescence-based assay using large unilamellar proteoliposomes containing NBD-labeled PC (Extended Data Fig. 10e). Intriguingly, the scramblase activity of type-2 rhodopsin does not depend on the chromophore binding, and thus the apoprotein opsin itself also functions as a phospholipid scramblase. To examine whether *Ta*HeR and opsin have the scramblase activity, we performed the scrambling assay according to the reported protocol. Our results indicated that both *Ta*HeR and opsin lack the ability to scramble phospholipids, unlike type-2 rhodopsin or opsin (Extended Data Fig. 10f-h).

Supplementary References

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