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Mediation mechanism of tyrosine 185 on the retinal isomerization equilibrium and the proton release channel in the seven-transmembrane receptor bacteriorhodopsin

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ABSTRACT

Electrostatic coupling leading to conformational changes in proteins is challenging to demonstrate directly, it requires that both the local, discrete electronic details and dynamic information relevant to the functional descriptions are probed. Here, as a novel study to address this challenge, the roles of an aromatic residue in influencing the functional conformational changes of a membrane receptor in its natural membrane environment are reported. Previously intractable discrete electronic details have been obtained using 2D solid-state NMR of specifically labelled receptor, reinforced with molecular dynamic simulations, mutational analysis and functional assays, supported by and compared with rigid-atom crystal structural models. Hydrogen bonding and hydrophobic interactions are identified as the mechanistic origin for direct electromechanical coupling to the dynamics of conformational changes within the receptor.

1. Introduction

Bacteriorhodopsin (bR), a member of the microbial rhodopsin family of seven-transmembrane (TM) receptors, functions as a light-driven proton pump for light energy capture in *Halobacterium salinarum* [1]. The retinal (Ret) chromophore is covalently bound to Lys216 on helix G to form a protonated Schiff base (SB) [2]. In the dark, two Ret isomers, 13-*cis*, 15-*syn* Ret (bR_{cis}) and all-*trans*, 15-*anti* Ret (bR_{trans}), are thermally interconvertible with a molar ratio close to 1:1 [3–6]. Conversion of the *cis* isomer to the all-*trans* configuration under illumination is known as light adaptation [6,7]. Absorption of a photon causes photoisomerization of the chromophore from the all-*trans* to the 13-*cis*, 15-*anti* configuration and triggers a series of structural rearrangements in the protein that initiates the vectorial translocation of a proton out of the cell [8].

The *cis-trans* thermal equilibrium of the Ret chromophore occurs in many bacterial rhodopsins in their native membrane, including bR [3], Neurospora Rhodopsin [9], Proteorhodopsin [10], Anabaena Sensory Rhodopsin [11], Channelrhodopsin-2 [12], Halorhodopsin [13], Xanthorhodopsin [14], Archaerhodopsin I-III [15–17]. However, the mechanism by which this equilibrium is maintained is not well described, and the direct demonstration of electrostatic coupling leading to conformational changes within proteins that explore multiple equilibria of states, is still challenging.

In bR, there are 26 amino acid residues contributing to the proton translocation channel in five helices, and 21 residues surround the Ret to form a binding pocket by all seven helices. Among them, 10 residues located in the middle of the proton translocation channel are also part of the Ret binding pocket [18]. Methionine 145 (M145), one of the residues contributing to the binding pocket on helix E, was the first residue identified to affect the *cis-trans* thermal equilibrium of the Ret chromophore in the dark [15]. The M145F mutation was shown to reduce the motional freedom of the tryptophan 182 in contact with the C13 methyl group of the Ret and to further destabilize the L state of bR [19]. On the other hand, tyrosine 185 (Y185), a binding site residue, was reported to play an important role in stabilizing the pentagonal hydrogen-bond network on the extracellular side of the retinal-lysine216 Schiff base (SB), and involved in the reprotonation of the SB during the early stages of the photocycle [20–23]. Furthermore, a ^{13}C solid-state NMR (ssNMR) study of [4′-^{13}C]-labelled tyrosine showed that the 11 tyrosine residues are protonated over a wide pH range from 2 to 12 in the dark-adapted bR [24]. Similarly, two different but co-existing conformations of Y185, based on a 4.3 ppm splitting of the ^{13}CO chemical shift of Y185, were observed [25,26]. The thermal conversion between the all-*trans* and 13-*cis* configuration of Ret in the dark-adapted purple membrane was

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considered to be the cause of the Y185 conformational change [25]. However, the crystal and solution NMR structures of bR_{trans} and bR_{cis} showed no displacements of Y185 during dark-light adaptation [27–29]. Therefore, the roles of Y185 in the bR photoreaction mechanism require further clarification with respect to the thermal isomerization equilibrium of the Ret chromophore, and conformational modulation of proton release during the bR photocycle.

In this study, the roles of Y185 in influencing the functional conformational changes of bR in its natural membrane environment are reported. Two-dimensional (2D) magic-angle spinning ssNMR of specifically labelled bR, reinforced with light-induced transient absorption measurements, molecular dynamics (MD) simulations and site-directed mutagenesis was used to examine how Y185 interacts with the Ret chromophore and modulates the proton release channel through aspartic acid 212 (D212) in its natural membrane environment. We first show that Y185 both maintains the *cis-trans* isomerization thermal equilibrium of the Ret chromophore, and retains aspartic acid 212 in a favorable conformation through a hydrogen bond to produce a cascade of conformational changes associated with photoreactions with the appropriate lifetime. Removal of the phenolic hydroxyl group in the Y185F mutant shifts the *cis-trans* isomerization thermal equilibrium to a bR_{cis}-dominated state and causes a weakening of the M state, loss of the O state and elongation of the proton pump cycle time.

2. Material and methods

2.1. Synthesis of ^{13}C-labelled all-trans-retinal

[10, 11-$^{13}C_2$]-, [14, 15-$^{13}C_2$]- and [10, 11, 14, 15-$^{13}C_4$]-labelled Ret molecules were synthesized by the standard method developed by Groesbeek and Lugtenburg [30]. The ^{13}C-labelled all-*trans*-Ret was purified by preparative HPLC using a 10 μm Luster Silica gel column (250 × 10.0 mm). Purity of the ^{13}C-labelled all-*trans*-Ret was examined by ^{1}H NMR on a 500 MHz spectrometer, and the isotope enrichment was better than 99%. The synthetic routes of the ^{13}C-labelled Ret are illustrated in Fig. S1 in the Supplementary material.

2.2. Protein sample preparations

Wild-type bR (WT-bR) purple membranes and Y185F mutant (Y185F-bR) were cultured and isolated by using either *H. salinarum* strain R1M1 or *H. salinarum* strain L33, and a sucrose gradient with concentrations of 35%, 43% and 60% (w/w) was used for purification of the purple membrane, according to previously described standard procedures [31]. The concentration of the purified bR was determined based on the absorption maximum at 568 nm using an extinction coefficient of 62,700 ± 700 M^{-1} cm^{-1}[32]. ^{13}C- and ^{15}N-labelled wild-type and Y185F mutant purple membranes were prepared by growing R1M1 and L33 in the synthetic media separately, in which the unlabelled tyrosine was replaced by the isotope-labelled one [33].

2.3. Incorporation of ^{13}C-labelled all-trans-Ret into the bR purple membrane

^{13}C-labelled-all-*trans*-Ret was incorporated into the bR purple membrane *via* bleaching and regeneration as previously described [34,35]. Briefly, the bR purple membrane was first completely bleached by illumination of the suspension in 50 ml of 0.5 M hydroxylamine at pH 7.0 for 3 h with a 550 nm cutoff filter; the bleached sample was then washed three times with 10 mM HEPES buffer (pH 7.0), and the ^{13}C-labelled-all-*trans*-Ret (1.2 M ratio excess) was slowly added in the dark with vigorous shaking at room temperature. Finally, the mixture was incubated at 4 °C in the dark for 24 h and then washed five times with 2% BSA to remove the unincorporated free Ret. The incorporation yield was approximately 80%.

2.4. UV–vis spectroscopy

UV–vis spectra were recorded using a T6 New Century spectrophotometer (Beijing Purkinje General Co. Ltd). The bR purple membrane was kept in the dark at room temperature for 2 h to ensure a fully dark-adapted form. The dark-adapted UV–vis measurement was then taken in the dark. The samples were then illuminated for 5 min to convert the dark-adapted form into the light-adapted form, then the light-adapted UV–vis spectrum was recorded.

2.5. Light-induced transient absorption change spectroscopy

The proton pumping activities of bR were monitored through a light-induced absorption change using a pH-sensitive dye, pyranine, on a homemade apparatus as described previously [36]. The net proton pumping activity was determined by taking the absorbance difference at 456 nm before and after adding the dye. The kinetics of the M state, O state and recovery trajectory to the ground state were monitored at 410 nm, 660 nm and 570 nm, respectively. All experiments were carried out by using a photoflash with the half-bandwidth less than 1 ms for excitation. All the measurements were performed at room temperature.

2.6. Solid-state NMR experiments

All solid-state NMR measurements were performed on either a Bruker 600 MHz Avance III wide bore spectrometer at −25 ± 1 °C or on a Bruker 850 MHz Avance III wide bore spectrometer at −65 ± 1 °C. A 3.2 mm or a 4.0 mm probe configured either in a double resonance or a triple resonance mode was used with a MAS spinning frequency of 8–16 kHz for different experiments. In all experiments, a ramped cross-polarization (CP) [37] with a 90%–100% linear gradient was used at a radio frequency (rf) field of 50 kHz on the proton channel. Typical 90° rf pulse lengths of 3.8 μs for ^{13}C and 2.7 μs for ^{1}H channels and the two-pulse phase modulation (TPPM) [38] with a pulse width of 5.5–6 μs for proton decoupling were used throughout the experiments. A recycle delay of 3 s was set for all the experiments. Detailed experimental setups for different 2D experiments are shown in the Supplementary material.

2.7. Molecular dynamic simulations

The POPC membrane was treated using the General Amber force field model for lipids [39]. The system contains 239 phospholipid molecules, with each phospholipid molecule consisting of 134 atoms. Asp96, Asp115, Glu204 and the SB in the inactive state and Asp85, Asp96 and Asp115 in the M state were protonated. To parameterize bR, the electrostatic potentials of both the ground and M states were calculated using Gaussian 09 [40] with the 6-31G* basis set and then used to obtain the restrained electrostatic potential (RESP) [41] fit of retinal-Lys Schiff base. Dihedral torsion potential parameters of both protonated and neutral Ret were achieved based on the work of Tajkhorshid [42], except for the parameter C12=C13–C14=C15, which was achieved based on the work of Hayashi et al. [43]. MD simulations were performed using the Gromacs dynamics software package [44], with the total simulation time set to 1 μs and the integration step set to 2 fs (leap-frog algorithm). The Nose-Hoover thermostat method [45] and Parrinello-Rahman method [46] were employed to maintain a constant temperature (300K) and pressure (1 bar). The particle mesh Ewald (PME) method [47] was applied to treat the long-range Columbic interactions.

3. Results

3.1. Activity assessments of the reconstituted ^{13}C-all-trans-Ret bR purple membrane

Reconstituted bR was shown to be functionally active through UV–vis spectroscopy and light-induced transient absorption measurements,