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Research paper

Comparative evaluation of the stability of seven-transmembrane microbial rhodopsins to various physicochemical stimuli

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ABSTRACT

Rhodopsins are seven-transmembrane proteins that function as photoreceptors for a variety of biological processes. Their characteristic visible colors make rhodopsins a good model for membrane-embedded proteins. In this study, by utilizing their color changes, we performed comparative studies on the stability of five microbial rhodopsins using the same instruments, procedures and media. As denaturants, we employed four physicochemical stimuli: (i) thermal perturbation, (ii) the water-soluble reagent hydroxylamine, (iii) the detergent sodium dodecyl sulfate, and (iv) the organic solvent ethanol. On the basis of the results, models for stabilization mechanisms in rhodopsins against each stimulus is proposed.

1. Introduction

Biological membranes form a boundary between the inside and outside of cells, preventing the leakage of cellular contents and acting as selectively permeable barriers with their membrane-embedded proteins in a stimulus-dependent manner. Membrane proteins also respond to environmental and intracellular signals as transmembrane receptors, channels and transporters. The secondary and tertiary structures of biological molecules, including membrane proteins, are maintained by a variety of physicochemical interactions, including electrostatic interactions, van der Waals contacts, hydrogen bonding, stacking and general non-polar contacts, all of which lead to reducing the total Gibbs free energy. Among the membrane proteins, seven-transmembrane (7-TM) proteins such as G protein-coupled receptors (GPCRs) play significant roles in producing membrane potential and transducing environmental stimuli into cells [1]. Their biological significance has made them critically important drug targets [1]. However, their instability, especially in the detergent-solubilized state, often hampers their structural and functional analysis.

Rhodopsins are photoreceptor proteins that have a chromophore retinal (vitamin-A aldehyde), which binds to a conserved lysine residue within the 7-TM α-helices via a protonated Schiff base [2]. Among rhodopsins, four microbial ones, bacteriorhodopsin (BR), halorhodopsin (HR), sensory rhodopsin I (SRI) and sensory rhodopsin II (SRII), which were identified from the archaeon *Halobacterium salinarum* (formerly *halobium*), have been extensively studied by a variety of methods to understand their molecular mechanisms of ion transport and signal transduction [2]. Since 2000, new types of rhodopsins have been found in various microorganisms due to advances in genomic technologies (Fig. 1A) [3]. Among them, we identified a microbial rhodopsin from the extreme thermophile *Thermus thermophilus* JL-18 in 2013 and named it thermophilic rhodopsin (TR) [4]. As expected, TR showed much a higher thermal stability than the other known rhodopsins [4]. Noteworthy, the high-resolution structures of microbial rhodopsins, including BR [5], HR [6,7], SRII [8], and TR [9], are quite similar (Fig. 1B), indicating that protein stability is regulated by differences in the detailed structures including amino acid side chain(s), ions and water molecules. Thus, in spite of their structures being very similar, the question arises as to how these rhodopsins maintain their thermal stability differently? To understand that, comparative and comprehensive studies among rhodopsins should be effective. It should be noted that the activities of rhodopsins are easily judged by color changes from orange, red and blue (490–580 nm) to yellow (360–420 nm), as shown in Fig. 1C. The chromophore retinal located in the middle of the 7-TM domain absorbs light at different wavelengths due to differences in the electronic energy gap between its ground- and excited states [10]. Variations in the wavelength of maximal absorption (λ_{max}) of rhodopsins arise due to interactions between the apoprotein (opsin) and the retinal chromophore [2,10]. Thus, the visible colors and their changes are good indicators for monitoring protein stability. In this study, we focused on five microbial rhodopsins whose functions and origins are different from each other; TR from

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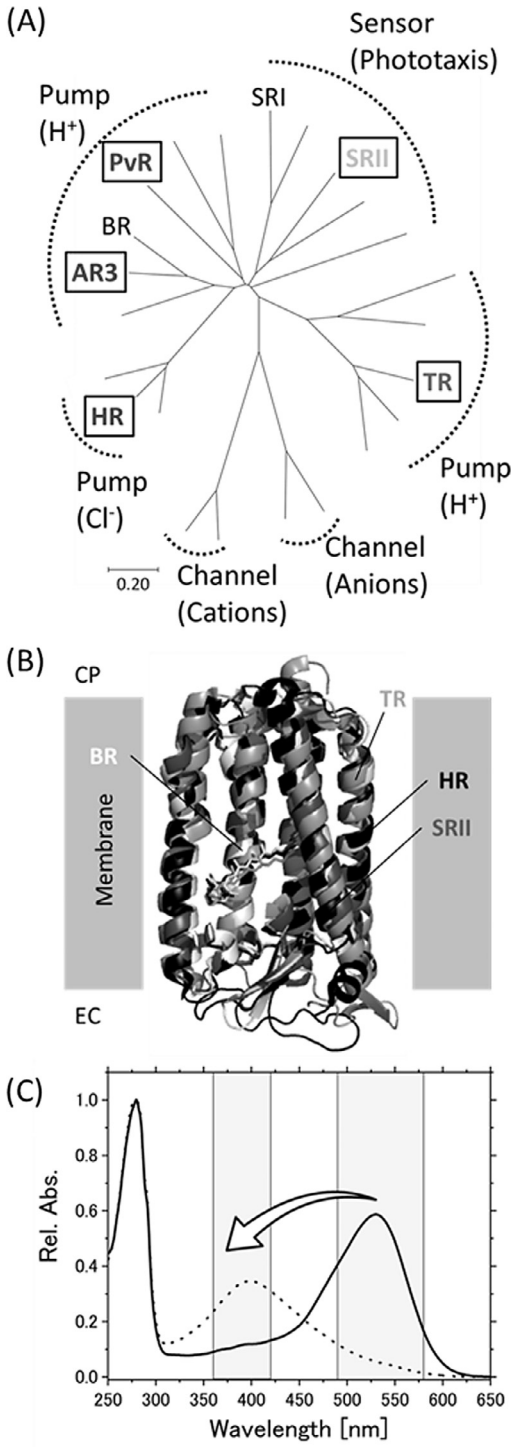

Fig. 1. Overview of seven-transmembrane microbial rhodopsins. (A) Unrooted phylogenetic tree of microbial rhodopsin amino acid sequences. All amino acid sequences were obtained from a public data base (https://www.ncbi.nlm.nih.gov) and aligned by MUSCLE program (http://www.ebi.ac.uk/Tools/msa/muscle/). Five microbial rhodopsins were used in this study: TR, SRII, HR, AR3 and PvR. Phylogenetic tree was constructed using the neighbor-joining method in MEGA7 program [27]. The scale bar indicates the number of amino acid substitutions per site. (B) Superimposed structures of microbial rhodopsins, BR (white, PDB code: 1C3W), TR (light grey, PDB code: 5AZD), SRII (dark grey, PDB code: 1H68) and HR (black, PDB code: 3A7K). CP and EC indicate cytoplasmic region and extracellular region, respectively. To visualize the retinal chromophore embedded in the proteins, the parts of some helices are removed from the structures. (C) SDS-induced spectral shift from native to denatured states of TR. The dotted and solid lines show UV–Vis spectra of purified TR with or without 3 w/v% SDS, respectively, in 50 mM Tris-HCl (pH 7.0) buffer containing 1 M NaCl and 0.05 w/v% DDM.

the bacterium *T. thermophilus* [4], SRII (also called phoborhodopsin, pR) and HR from the archaeon *Natronomonas pharaonis* [11,12], archaerhodopsin-3 (AR3) from the archaeon *Halorubrum sodomense* [13] and Pantoea vagans rhodopsin (PvR) from the bacterium *Pantoea vagans* [14]. Those five rhodopsins have advantages since they can be functionally expressed in *Escherichia coli* as recombinant proteins and all of them have predominantly all-*trans* retinal in their protein moiety as a chromophore [4,14–17]. We investigated and compared their stability against four different physicochemical stimuli: (i) thermal perturbation as an indicator of overall physicochemical interactions inside proteins, (ii) a water-soluble reagent hydroxylamine (HA) as an indicator of hydrophilicity of the extracellular region, (iii) a detergent sodium dodecyl sulfate (SDS) as an indicator of the stability of the folding core, and (iv) an organic solvent ethanol (EtOH) as an indicator of internal water molecules, by means of time-dependent changes of their colors.

2. Materials and methods

2.1. Sample preparation

The expression plasmids for the histidine-tagged TR, SRII, HR, AR3 and PvR were constructed as described previously [4,14,15,18,19]. The *E. coli* strains, DH5α and BL21(DE3), were used as hosts for DNA manipulation and for protein expression, respectively. Protein expression, preparation of the crude membranes and protein purification were performed using essentially the same method as described previously [4]. In short, *E. coli* BL21 (DE3) cells harboring the plasmids were grown at 37 °C in 50 mL LB medium supplemented with ampicillin (final concentration, 50 μg mL^{-1}) and protein expression was induced at an OD_{660} of 1.4–1.6 with 1 mM isopropyl β-D(-)-thiogalactopyranoside (IPTG) and 10 μM all-*trans* retinal (Sigma-Aldrich, St. Louis, MO, USA). After induction for 4 h, the rhodopsin-expressing cells were collected by centrifugation at 4 °C and were then disrupted by sonication (UD-211, TOMY Seiko Co., Ltd., Tokyo, Japan) on ice-cold water. The crude membranes were collected by ultracentrifugation and were then solubilized with 1.5 w/v% n-dodecyl-β-D-maltoside (DDM, Dojindo Lab., Kumamoto, Japan) in 50 mM Tris-HCl (pH 8.0) buffer containing 300 mM NaCl. After ultracentrifugation again, the supernatant was applied to a HisTrap FF Ni^{2+}-NTA affinity chromatography column (GE Healthcare, Amersham Place, England) which was previously equilibrated with the same buffer using an ÄKTA purifier chromatography system (GE Healthcare). The Ni^{2+}-NTA resin in the column was washed sufficiently with 50 mM Tris-HCl (pH 8.0) buffer containing 1 M NaCl, 0.1 w/v% DDM and 20 mM imidazole. Proteins were eluted by increasing the concentration of imidazole, and were then concentrated by centrifugation using an Amicon Ultra filter (30,000 MW cutoff; Merck Millipore, Bedford, MA, USA). The sample medium was replaced by the appropriate buffer solution by ultrafiltration for more than 5-times.

2.2. Thermal stability

Before the measurement, the purified proteins were dialyzed against buffer-A (1 M NaCl, 50 mM Tris-HCl, 0.05 w/v% DDM, pH 7.0) for more than 2 weeks to properly control the concentration of DDM. Time-dependent thermal denaturation at 75 °C was monitored using a UV2450 spectrophotometer (Shimadzu, Kyoto, Japan) every 0.5 nm. During incubation, the suspension became turbid because of the aggregation of denatured proteins. Therefore, before the spectrum measurement, sample proteins were cen-