Crystal structure of the proton pumping rhodopsin ARII from marine alga *Acetabularia acetabulum*

The membrane protein rhodopsins, consisting of opsin with seven helices and a retinal chromophore, can be classified in three types according to their electrical properties: 1) ion pump, including the proton pump of bacteriorhodopsin (BR) from the archaeon *Halobacterium salinarium* and the chloride pump of *Natronomonas* halorhodopsin (NpHR); 2) channel rhodopsin, such as the cation channels from *Chlamydomonas* (ChR1,2); and 3) electrically neutral rhodopsin, such as the visible photoreceptor in the human retina and *Anabaena* sensory rhodopsin (ASR). The best-studied rhodopsin is BR, in terms of its proton transport mechanism. BR at the ground state, and the intermediates K, L, M, N and O during the photocycle, have been analyzed by spectroscopic methods and X-ray crystallography. Proton pumping rhodopsins have been widely found in not only archaea, but also bacteria and eukarya. The first proton pumping rhodopsin discovered in a green plant was the rhodopsin from the marine alga *Acetabularia acetabulum*. Recently, some algal rhodopsins have been applied in neurosciences. Neuron spiking in nerve cells expressing chloride and proton pumps, including NpHR and *Acetabularia* rhodopsin (AR), and the channel rhodopsins, including ChR2, can be controlled by laser light, even in the mouse brain. Furthermore, a modified NpHR, engineered for high level expression in neurons, was used for ultrafast optogenetics.

We now describe the crystal structure and the function of a newly identified AR, ARII [1]. ARII was synthesized by a cell-free protein synthesis system with detergent/lipid [2], and was crystallized by the *in meso* method, using monoolein. Many reddish-purple, plate-like crystals (Fig. 1) grew to sizes of 100×100×10 μm^3 at 20°C in two weeks. The ARII crystal was picked from the lipidic mesophase and flash-cooled in a nitrogen stream at –180°C, as soon as the seal of the glass plate was opened. Data collection was performed using a 10 μm-size microbeam at **BL41XU** beamline. The crystals were unstable under the open air conditions, and were easily damaged during handling with the micro tool. Thus, the crystal-picking and diffraction check were performed repeatedly, until diffraction pattern images that could be indexed were obtained. The crystal structure of ARII, consisting of seven helices (A to G) and a retinal molecule, was determined at 3.2 Å resolution (Fig. 2). Four ARII proteins with eight cholesterol molecules were observed in the asymmetric unit. A structural alignment (Fig. 3) performed by the DALI server revealed that the ARII structure is quite similar to those of other rhodopsins, such as BR, *Natronomonas pharaonis* sensory rhodopsin II (NpSRII), and ASR. The highly conserved regions of helix C and the C-terminal portion of helix F might participate in a common mechanism for ion-pumping and photo-sensing by rhodopsin, because the local flex of helix C at the L-intermediate and the outward movement of helix F at the M2-intermediate have been observed in BR and NpSRII. In helix G, the carbonyl groups of Val208 and Leu209 form hydrogen bonds with the amide NH groups of Leu213 and Asn214, respectively. These interactions create a π-bulge and kink the helix at Lys211, which is bound with the all-*trans* retinal chromophore and forms a Schiff base.

At the transition from the L to M state in the BR

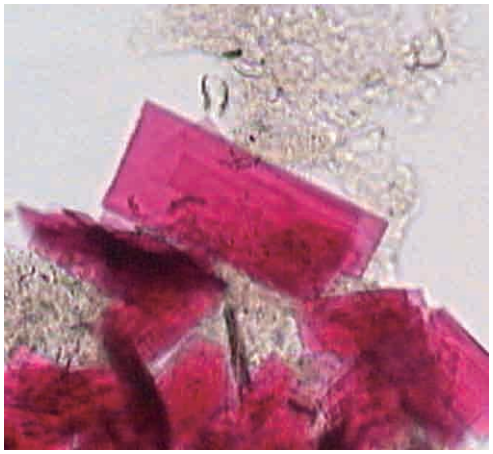

Fig. 1. Crystals of ARII. The reddish-purple plate-like crystals were grown in a monoolein mesophase.

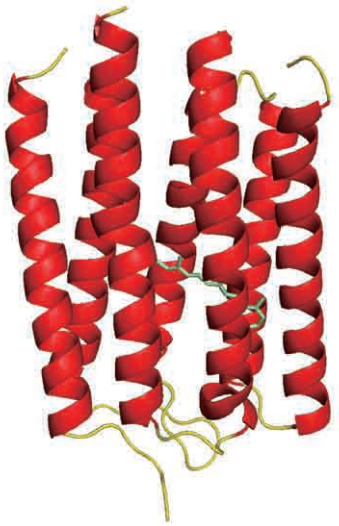

Fig. 2. Crystal structure of ARII. The seven α-helices and the loops in the opsin are colored red and yellow, respectively. The retinal molecule is light green.

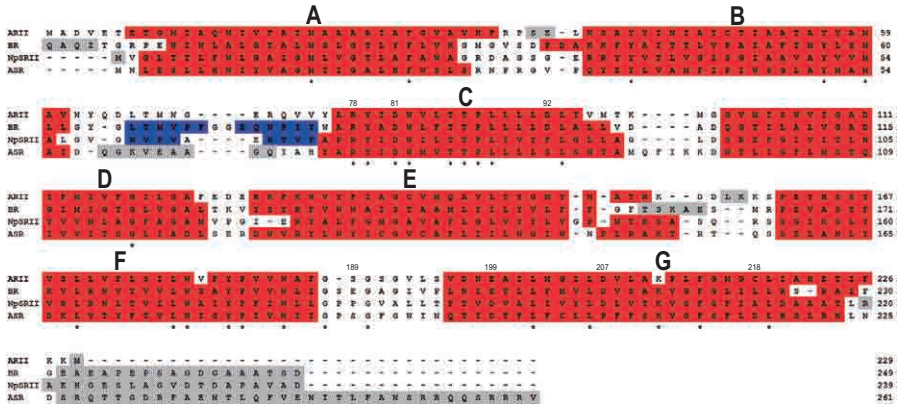

Fig. 3. Structure-based sequence alignment of ARII with BR, NpSRII, and ASR. The α-helices, β-strands, and undetermined regions are shown in red, blue, and gray, respectively. The identical residues in the sequence alignment are marked by asterisks. The residue numbers are provided in the column on the right side of the alignments, and above the ARII residues involved in the proton transfer.

photocycle, a proton is transferred from the protonated Schiff base to the carboxyl oxygen atoms of Asp85. The Schiff base is hydrogen-bonded to a water molecule, which forms a hydrogen-bond network with Asp85, Asp212, Arg82, and two water molecules. A proton is then released to the extracellular space from the proton release group, consisting of Arg82, Glu194, and Glu204. Recently, it was suggested that Glu194 and Glu204 in BR are bonded by a delocalized proton, and a low-barrier hydrogen bond might be advantageous for rapid proton release. In ARII, rapid proton release does not occur at neutral pH. We compared the structure of the proton release pathway in ARII with those in BR and NpSRII. The structure-based alignment diagram (Fig. 3) indicated that Asp85, Asp212, Arg82, Glu194, and Glu204 in BR correspond to Asp81, Asp207, Arg78, Ser189, and Glu199 in ARII, and Asp75, Asp201, Arg72, Pro183, and Asp193 in NpSRII. The superposition of the crystal structure of ARII onto that of BR revealed that the side chains of these residues fit well with those of BR. However, the orientation of the Arg78 side chain is opposite to that of the corresponding Arg82 of BR (Fig. 4), but the same as that of Arg72 of NpSRII, which is capable of proton transfer. In BR, proton release is triggered by the movement of the Arg-82 side chain toward the glutamate pair upon the formation of the M intermediate, causing the p*K*a of the proton releasing residue to dramatically change (9.7 to 5.7). In NpSRII and ARII, this Arg side chain does not move, since its guanidium moiety is oriented toward $Asp193^{NpSRII}$ or $Glu199^{ARII}$ at the ground state. The pKa of Asp193 in NpSRII is 6.4 at the ground state, and 4.9 at the M-intermediate, and the rapid proton release occurs at neutral pH, unlike ARII. No electron density was observed between the ε1 oxygen atom of Glu199 and the η1 nitrogen atom of Arg78 at a 3.9 Å distance in the map. Therefore, a water molecule may not exist around Glu199 and Arg78 in ARII. This may disrupt the formation of the low-barrier hydrogen bond at Glu199, resulting in the "late proton release" observed by biochemical analyses, such as proton transfer measurements with the ITO electrode and flash-photolysis. This is the first report on the crystal structure of a eukaryote-derived microbial rhodopsin, synthesized functionally by a cell-free synthetic method.

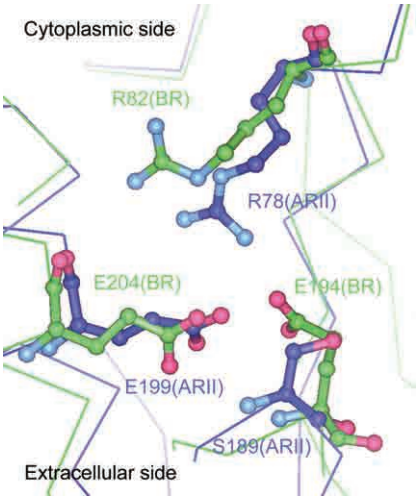

Fig. 4. Amino acid residues involved in the proton transfer. Superimposition of the ARII and BR structures, colored blue and green, respectively.

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