Rapid Communication in Photoscience(2013), vol. 2, no. 2, pp.60-63
http://dx.doi.org/10.5857/RCP.2013.2.2.60
ISSN 2234-8182
eISSN 2288-4564

Communication

Characterization of a Xanthorhodopsin-homologue from the North Pole

Se Hwan Kim[1], Jang-Cheon Cho[2] and Kwang-Hwan Jung[1*]

[1] Dept. of Life Science and Institute of Biological Interfaces, Sogang University, Seoul, 121-742, Korea
[2] Dept. of Biological Sciences, Inha University, Incheon 402-751, Korea

ABSTRACT : Rhodopsins belong to a family of membrane-embedded photoactive retinylidene proteins. One opsin gene was isolated from β-proteobacterium (IMCC9480) which had been collected at the North Pole. It is very similar to Xanthorhodopin (XR) of HTCC2181. In this study, we carried out basic characterization of the rhodopsin. It has λmax of 536, 554, and 546 nm at pH 4.0, 7.0, and 10.0, respectively. Since the pKa of its proton acceptor is around 6.27, we measured its proton pumping activity and photocycling rate at pH 8.0. It has a typical proton acceptor (D99) and donor (E110) which mediate proton translocation from intracellular to extracellular region when deduced from the sequence alignments. On the basis of *in vitro* proton pumping activity, it was proposed to have fast photocycling rate with M and O intermediates, indicating that it is a typical ion-pumping rhodopsin. Since the XR has not yet been expressed in any other heterologous expression system, we tried to get much more information about the XR through the XR-homologue rhodopsin.

Rhodopsins belong to a family of membrane-embedded photoactive retinylidene proteins. They share a common structure of seven transmembrane helices forming an interior pocket for chromophore retinal. Rhodopins are classified into two groups based upon their primary sequences. Type I or microbial rhodopsin is an archaeal-type rhodopsin first discovered in *Halobacterium salinarum*, halophilic archaea that contains bacteriorhodopsin(BR), halorhodopsin(HR), and two receptors mediating phototaxis, called sensory rhodopsins I and II(SRI and SRII).

***To whom correspondence should be addressed.**
E-mail: kjung@sogang.ac.kr

Archaeal-type (Type I) rhodopsins have been extensively studied as a model system that both explain how light energy converts to chemical potential, and how environmental signals relay and change the bacterial phototactic behavior. They play two roles: light-driven ion transporters or photosensory photoreceptors.

Xanthorhodopsin is one of proton pumping rhodopsins, which is a retinal protein/carotenoid complex in the eubacterium *Salinibacter ruber*. These cells contain an unusual retinal protein, which uses salinixanthin to assist harvesting light energy in a wider spectral range and utilizes it for transmembrane proton transport.

Rhodopsin homologue genes were amplified from genomic DNA samples of IMCC9480 (β-proteobacterium) which had been isolated from a surface seawater sample collected off the coast near the Dasan Korean Arctic Station in Ny-Ålesund, Svalbard, Norway (79°00′07″N, 11°18′33″E) by PCR (Polymerase Chain Reaction) using conserved primers and degenerate primers. After multiple sequence alignment analysis, we revealed that IMCC9480 had new rhodopsin homologue that has high homology with XR(Fig. 1A). The sequence encoded protein, referred to as the IMCC9480 opsin, and contained all the significant amino acid residues essential for retinal binding. Interestingly, GPR type PRs were found at the Arctic Ocean, while BPR types were identified from the Antarctic Ocean. In this study, new XR type gene was isolated from samples collected from the Arctic ocean. Purified IMCC9480 rhodopsin was found to display absorption maximum of 554, 536 and 542 nm at pH 4, 7 and 10, respectively (Fig. 1B).

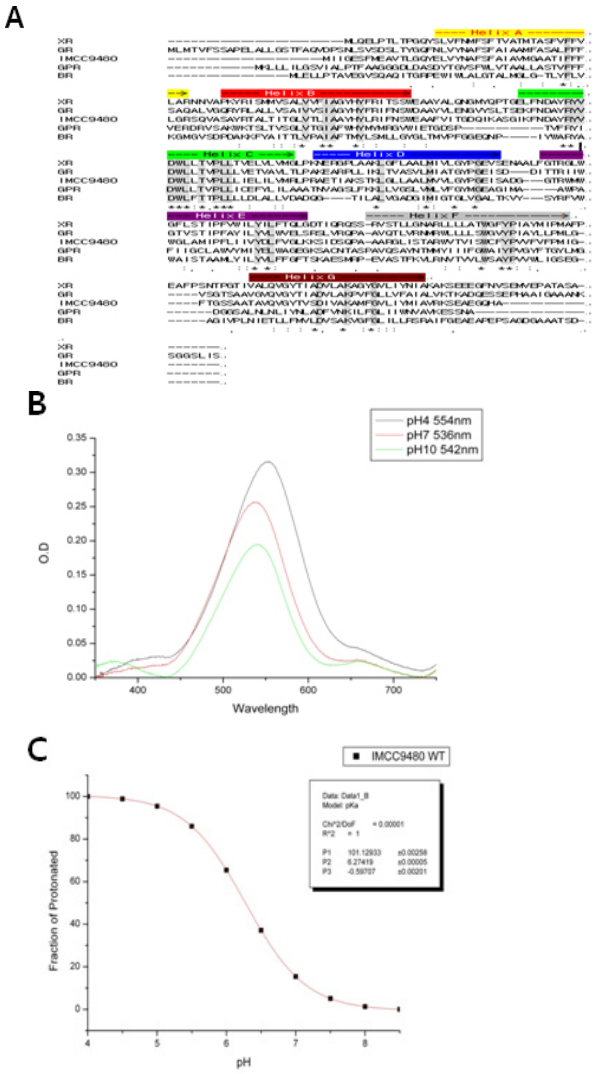

Figure 1. Alignment of IMCC9480 rhodopsin and its pH-dependent absorption spectrum. (A) Alignment of IMCC9480 WT rhodopsin with several pumping rhodopsins. It shows high similarity to GR and XR. (B) Absorption maxima of 554, 536 and 542 nm at pH 4, 7 and 10, respectively. (C) pKa value of IMCC9480 rhodopsin. It has a pKa value of 6.27.

In general, the spectra of rhodopsins exhibit two forms at a proton-dependent equilibrium: alkaline species in which the primary retinylidene Schiff base counterion (Asp97 in PRs) is unprotonated, and a red-shifted acidic species in which the residue is protonated. When we obtained the absorption spectra of IMCC9480 at several pH, we could identify one isosbestic point. Based on the results, we could calculate a pKa value of IMCC9480 to be 6.27(Fig. 1C). The pH value of sea water usually lies between 7.4 ~ 8.1, and in those conditions, the Schiff base of this protein remains in deprotonated state for the most of time in natural conditions.

Following the analysis of its basic optical characteristics, we turned to measure light-induced difference spectrum of IMCC9480, leading to the identification of light-induced functional intermediates. There were two forms of intermediates at around 400 nm and 600 nm. The one at 400 nm was found to be M intermediate and the other at 600 nm O intermediate (Fig. 2A). One negative value at 550 nm represents ground state of IMCC9480 rhodopsin. Furthermore, we performed laser-induced difference spectroscopy to measure decay rate of each intermediates. Absorbance changes were measured by using RSM 1000 (Olis, USA) with laser flash emitted from a Nd-YAG pulse laser. M intermediate showed fast decay rate of 1.5 msec (Fig. 2B). Such a rate is similar to those of other ion transporting rhodopsins. Ion transporting rhodopsin usually displayed fast M intermediate decay below 5msec. O intermediate also showed pretty fast decay rate, corresponding to 7.1msec (Fig. 2B). The value is also similar to those of other ion transporting rhodopsins. In general, ion-transporting rhodopsins, including IMCC9480, showed rapid photocycling rate. In order to understand the proton pumping efficiency of IMCC9480, we tried to measure the ion transporting activity by using pH electrode. It was evident from the previous alignment analysis that IMCC9480 was very close to GR (*Gloeobacter* rhodopsin) and XR, which are known as outward proton pumping rhodopsins. We measured pH changes resulted from IMCC9480 rhodopsin in order to examine its proton transportation ability. Using the sphaeroplast preparations of *E. coli,* we monitored pH changes of the preparations influenced by the shift of light illumination at a 60sec interval. We measured the pumping activity with pH conditions over pH 8.0, which lies in the pH range of sea water. As we expected, IMCC9480 showed active proton transport. With elapse of time, pH decreased when the light was given, while it was restored to the original value when the light is off (Fig. 2C), indicating that the protein is able to transport protons from the inside of the cell to the outside during light illumination.

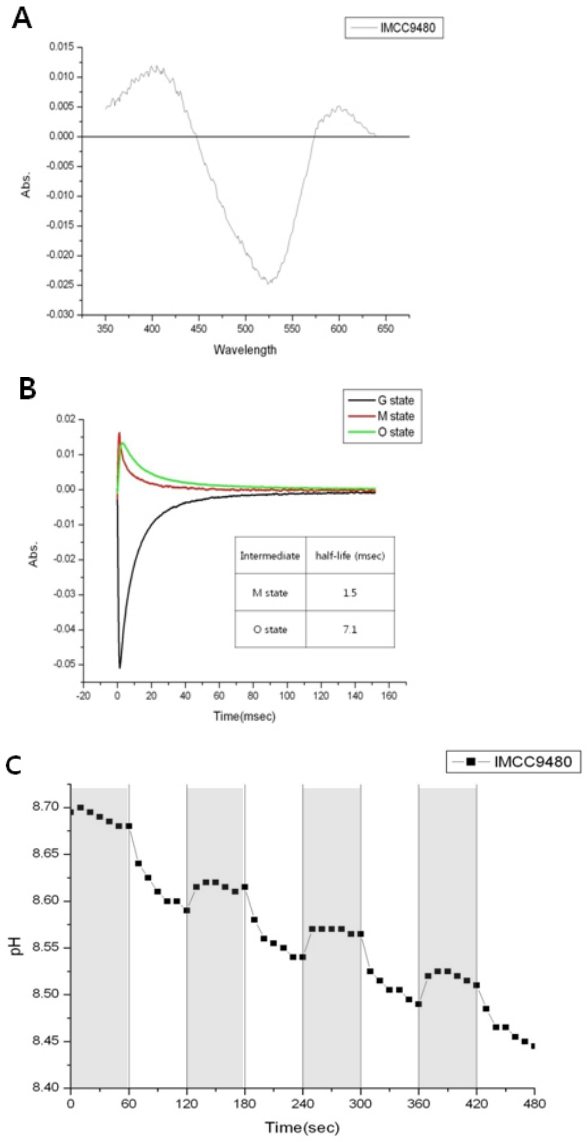

Figure 2. (A) Two forms of IMCC9480 intermediates. M intermediate is appeared around at 400 nm, while O intermediates at around 600 nm, as revealed by light-induced differential spectrum analysis. (B) Rapid decay shown by M intermediate of IMCC9480, which is close to typical pumping rhodopsin. (M decay is 1.5 msec and O decay is 7.1 msec). (C) Outward proton-pumping activity of IMCC9480 under illumination, which manifests very clearly at around pH 8.0. (gray background: dark, white background: light)

The IMCC9480 rhodopsin also might have functions to energize cells by light–driven electrogenic proton pumping like other proton pumping rhodopsins. It would be helpful to make IMCC9480 cells survive better in the coastal seawater. Expression of XR has not been successful in any of other hosts because of the difficulties resided in the expression studies. We tried to obtain as much information about XR as we can by thorough examination of the XR-homologue.

Experimental Procedures

Collection of samples and extraction of DNA from the marine bacteria

Dr. Jang-Cheon Cho (Inha University, Korea) kindly provideed us metagenome samples from Dasan Korean Arctic Station in Ny-Ålesund, Svalbard, Norway (79°00′07″N, 11°18′33″E). Samples were collected from sea surface from the region. Total genomic DNA was extracted using a sterivex method and PBC method.

PCR amplification from the DNA samples from Arctic Sea

Metagenome samples were obtained from Arctic Ocean. It was used as a template for PCR to discover new types of rhodopsins. Primers were designed using conserved N-terminal and C-terminal regions of MBP (eBAC31A08), as well as conserved regions in Helix C and F. Non-degenerate primer, generate primer (non-degenerate primer; 5'-ATGAAANNATTANTGATNTT-3', generate primer; 5'-ATGAAATTATTACTGATATTAGG-3', reverse primer; 5'-AGCATTAGAAGATTCTTTAACAGC-3') and conserved primer (conserved primer; 5'-TTNMGNTAYATHGAYTGG-3', reverse conserved primer; 5'-CGGGTAAATCGCCCAACC-3') were used. PCR was performed with 35 cycles at 95 ℃ for 2 min, 47~51℃ for 2 min 30 sec, 72℃ for 2 min 30 sec. PR genes were amplified with *Taq* polymerase (Vivagen, Korea) and cloned into T-blunt vector (Solgent, Korea).

Expression and purification of xanthorhodopsin-like new rhodopsin from IMCC9480

The pKA001 plasmid contains a rhodopsin gene and a mouse *dioxygenase* gene which can convert β-*carotene* to two all-trans retinal. IMCC9480 opsin was cloned into the pKA001 and expressed in *Escherichia coli* strain UT5600 using retinal and pKA001. IMCC9480 opsin gene was under the lacUV5 promoter and mouse *dioxygenase* genes were under the P_{BAD} promoter to produce dioxygenase in *E. coli*. For photochemical measurements, UT5600 cells transformed with pKA001 were induced with 1 mM IPTG and 5~10 μM all-*trans* retinal (Sigma, USA) for 4~8 hrs

at 37℃. Expressed cells were suspended in sonication buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.0), sonicated for 4 min (15 sec pulse) (Branson sonifier 250), and the membrane fraction was treated 1% n-dodecyl-α-D-maltopyranoside (DDM) (Anatrace, USA). The solubilized fraction was incubated with Ni^{2+}-NTA agarose (Qiagen, USA) and eluted with 250 mM Tris-HCl (pH 7.0), 150 mM NaCl, and 0.02% DDM.

Measurements of absorption spectrum and pKa

Absorption spectroscopy was used to measure absorption maxima of the rhodopsins and pKas of the Schiff base counterion in purified IMCC9480 rhodopsin. The absorption spectra were recorded with UV/VIS spectrophotometer (UV-2550) (Shimadzu, Japan) at pH 4, pH 7, and pH 10. In order to calculate the pKas of the primary proton acceptor, the spectrum at pH 7.0 was used as a reference and pH was raised to pH 10.0 and lowered to pH 4.0. Collected data from different absorption spectra were determined and fitted with functions containing pKa components [$y=A/(1+10^{pH-pKa})$] using Origin Pro 7.0.

Preparation of E. coli sphaeroplasts and measurement of the proton pumping activity

250 mL of rhodopsin-expressed cells was centrifuged and suspended in 10 mL of plasmolysis buffer (30 mM Tris-HCl, pH 8.0, and 20% sucrose) with lysozyme (Usb, USA). Sphaeroplasts were collected and resuspended in 400 μL DNA lysis buffer (100 mM KPi, pH 7.0, 20 mM $MgSO_4$, 20% sucrose, 1.6 mg DNase I) and injected using a 1 mL syringe (26 gauge needle) into 200 mL of rapidly shaking osmotic shock solution (50 mM KPi, pH 7.0). After shaking at 37℃ for 15 min, Na-EDTA was added to a final concentration of 10 mM and shaked for 15 min. $MgSO_4$ was added to a final concentration of 15 mM and shaked for another 15 min. Sphaeroplast vesicles were collected at 30,778 x g for 1 hr at 4℃ (Beckman XL-90 ultracentrifuge, USA) and washed with 10 ml unbuffered solution (10 mM NaCl, 10 mM $MgSO_4$, 10 μM $CaCl_2$). Finally, sphaeroplasts were resuspended in 3 mL of unbuffered solution. Samples were illuminated through the short wave cutoff filter (>440 nm, Sigma Koki SCF-50S-44Y, Japan) in combination with focusing convex lens and heat-protecting ($CuSO_4$) filter. The pH values were monitored (Horiba pH meter F-51) and pH data were transferred and recorded automatically with Horiba data Navi program.

Light-induced difference spectra and Laser induced absorption difference spectroscopy

Samples were illuminated through Cole-Parmer illuminator (41720 series) and time-resolved difference spectra were measured by S-3100 diode-array spectrophotometer (Scinco, Korea). Flash-induced absorbance changes were measured on RSM 1000 (Olis, USA) and laser flash was from a Nd-YAG pulse laser (Continuum, Minilight II, 532 nm, 6 ns, 25 mJ). 10-30 signals were averaged for measuring the rate of formation and decay of the photointermediates. IMCC9480 rhodopsin expressed *E. coli* membrane fractions suspended with 0.08% DDM in sonication buffer. Heavy membranes or cell debris were eliminated by centrifugation at 4,000 x g for 4 min. Light membranes were collected by ultracentrifugation at 40,000 x g for 20 min and then resuspended in DW. The membranes were embedded into 7% polyacrylamide gels which were soaked in 150 mM NaCl at pH 9.0 for measuring laser-induced absorbance difference kinetics of IMCC9480 rhodopsin.

KEYWORDS : β-proteobacterium, ion-transporter, rhodopsin,

Received June 17, 2013; Accepted July 30, 2013

ACKNOWLEDGEMENT

This work was supported by the Research Foundation of Korea grants (2011-220-C00057) and Ministry of Science ICT & Future Planning (20122055325)

REFERENCES AND NOTES

1. Spudich, J. L.; Yang,C. S.; Jung K. H.; Spudich E. N., *Annu. Rev. Cell Dev. Biol.* **2000**, *16*, 365-392.
2. Oh, H.M.; Lee K.Y.; Jang, Y.R.; Kang, I. N.; Kim, H. J.; Kang, T. W.; Kim, S. Y.; Cho, J. C. *J Bacteriol.* **2011**, 193(13), 3421-3427.
3. Kawanabe, A.; Furutani, Y.; Jung, K. H.; Kandori, H. *Biochemistry* **2006**, 45, 4362-4370.
4. Jung, J.Y.; Choi, A. R.; Lee, Y.K.; Lee, H.K; Jung, K. H. *FEBS-Letters*., **2008**, *582*, 1697-1684.
5. Balashov, S.P.; Imasheva, E. S.; Boichenko, V. A.; Anton, J.; Wang, J. M.; Lanyi, J.K. *Science.,* **2005**, *309*, 2061-2064.
6. Wang, W.W.; Sineshechekov, O.A.; Spudich, E. N.; Spudich, J. L. *J Biol Chem*, **2003**, *278(36)*, 33985-33991.