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Photochemical characterization of actinorhodopsin and its functional existence in the natural host

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ABSTRACT

Actinorhodopsin (ActR) is a light-driven outward H^+ pump. Although the genes of ActRs are widely spread among freshwater bacterioplankton, there are no prior data on their functional expression in native cell membranes. Here, we demonstrate ActR phototrophy in the native actinobacterium. Genome analysis showed that *Candidatus* Rhodoluna planktonica, a freshwater actinobacterium, encodes one microbial rhodopsin (RpActR) belonging to the ActR family. Reflecting the functional expression of RpActR, illumination induced the acidification of the actinobacterial cell suspension and then elevated the ATP content inside the cells. The photochemistry of RpActR was also examined using heterologously expressed RpActR in *Escherichia coli* membranes. The purified RpActR showed λ_{max} at 534 nm and underwent a photocycle characterized by the very fast formation of M intermediate. The subsequent intermediate, named P_{620}, could be assigned to the O intermediate in other H^+ pumps. In contrast to conventional O, the accumulation of P_{620} remains prominent, even at high pH. Flash-induced absorbance changes suggested that there exists only one kind of photocycle at any pH. However, above pH 7, RpActR shows heterogeneity in the H^+ transfer sequences: one first captures H^+ and then releases it during the formation and decay of P_{620}, while the other first releases H^+ prior to H^+ uptake during P_{620} formation.

1. Introduction

Microbial rhodopsins are ubiquitous membrane proteins in unicellular microorganisms [1,2]. They commonly consist of seven transmembrane helices surrounding a chromophore retinal, which binds to a conserved lysine residue in the last helix *via* a protonated Schiff base (PSB; the deprotonated Schiff base is abbreviated as SB). Upon illumination, the retinal undergoes isomerization from an all-*trans* to a 13-*cis* configuration. This change elevates the protein to the excited state, which is thermally relaxed to the original state *via* various photochemical intermediates. During this cyclic reaction, called the photocycle, microbial rhodopsin performs various roles, including light-driven ion pumps, ion channels, and photosensory transductions. In 1971, the first microbial rhodopsin was discovered in an extremely halophilic archaeon, *Halobacterium salinarum*, and named bacteriorhodopsin (BR) [3]. Subsequently, it was proven that BR acts as an outward H^+ pump and can drive cellular ATP synthesis by creating an H^+-electrochemical gradient across the cell membrane [4]. Later, three relatives with different functions were discovered from the same archaeon: inward Cl^- pump halorhodopsin [5], phototaxis sensors sensory rhodopsin I [6], and sensory rhodopsin II (also called phoborhodopsin) [7]. For approximately 30 years, research on microbial rhodopsins was confined to these four archaeal rhodopsins [8]. Since 1999, however, the genes for other microbial rhodopsins have been identified in various microorganisms, including eubacteria, fungi, algae, and even viruses. Some new members have the same functions as the archaeal rhodopsins, whereas others perform novel roles, acting as outward Na^+ pumps [9,10], cation and anion channels [11–14], and sensors for the regulation of gene expression [15]. Thus, microbial rhodopsins are now identified as a large and diverse family.

H^+ pumps define the largest functional class in the microbial rhodopsins and are widespread in various microorganisms inhabiting a broad range of environments. For aqueous environments, their abundances have been clarified through metagenomic analyses. For marine

Abbreviations: ActR, actinorhodopsin; RpActR, ActR from *Candidatus* Rhodoluna planktonica; PSB, protonated Schiff base; SB, deprotonated Schiff base; BR, bacteriorhodopsin; PR, proteorhodopsin; XR, xanthorhodopsin; GR, *Gloeobacter* rhodopsin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DDM, *n*-dodecyl β-D-maltopyranoside; PC, phosphatidylcholine; ITO, indium tin oxide; ORF, open reading frame; ESR, *Exiguobacterium sibiricum* rhodopsin; PRG, H^+-releasing group; EC, extracellular; CP, cytoplasmic.

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environments, the gene encoding the H^+ pump was first identified in 2000 in a DNA fragment from an uncultivated γ-proteobacterium (the SAR86 group), which is one of the most abundant marine bacterioplankton [16]. This H^+ pump was named proteorhodopsin (PR). The expression of photoactive PR was verified in native planktonic membrane preparations [17]. Later, PR genes were identified in other oceanic microbial groups, and it was established that PR-encoding microorganisms are common in the world oceans (for reviews, see [8,18]). The cellular expression of PR and its H^+-pumping activity were also proven in cultivated host strains. In some strains, light-activated PR conferred observable benefits on the cells, such as growth enhancement, fuel CO_2 fixation, and extended survival under starvation. Functional PR is available from the heterologous *Escherichia coli* system with exogenous retinal [16]. Thus, the photochemistry of PR has been examined in detail (for reviews, see refs [2,19,20]).

For non-marine aqueous environments, metagenomic analysis revealed that a PR-related but phylogenetically distinct gene group is present in high abundance [21]. This predicted H^+-pump group was named actinorhodopsin (ActR) because these genes are exclusively associated with actinobacteria, which are common inhabitants of freshwater environments [22]. Later, ActR genes were indeed found in cultivated freshwater actinobacteria [23] and further identified in other freshwater bacterioplankton [24]. Thus, it is now established that ActR genes are widespread in freshwater environments. In contrast to PR, ActR genes are almost completely absent in marine environments [21].

Despite its broad distribution, ActR has been less well characterized in both its physiological and photochemical aspects. Its broad distribution implies its significant contribution to the solar energy utilization in the environment. However, a previous study was not able to demonstrate the fully functional expression of ActR in the native cells. In actinobacterium *Rhodoluna lacicola* strain MWH-Ta8, the ActR opsin was constitutively expressed but did not contain the retinal [25]. This immature ActR required exogenous retinal to perform the H^+-pumping function. This result was consistent with the fact that *R. lacicola* lacks the gene encoding the homolog of β-carotene cleavage enzyme (β-carotene 15,15′-monooxygenase), which is necessary for the final step of the known retinal biosynthesis process.

In this study, we examined ActR from the freshwater actinobacterium *Candidatus* Rhodoluna planktonica strain MWH-Dar1. Hereafter, this ActR is designated RpActR. The genome analysis showed that this strain encodes the genes for retinal biosynthesis enzymes, including a putative β-carotene cleavage enzyme, and lacks microbial rhodopsin genes other than RpActR. Without the exogenous retinal, RpActR performed outward H^+-pumping activity in the native cell membrane and thus drove ATP production. Thus, this study includes the first observation of ActR phototrophy in native actinobacterial cells. RpActR was also photochemically characterized after heterologous expression in *E. coli* cells. The purified RpActR had a λ_{max} of 534 nm and underwent a photocycle characterized by the very fast formation of M intermediate and prominent accumulation of O-like intermediate (named P_{620}) even at high pH. Together with the observed H^+-transfer reactions, the photocycle of RpActR will be discussed.

2. Materials and methods

2.1. Cultivation and DNA sequence analysis of the actinobacterium

The actinobacterium was grown in 3 g/L NSY medium at pH 7.2 [26]. The culture of strain MWH-Dar1 contained initially a small fraction of non-actinobacterial contamination (<1%) [27]. In order to establish a pure culture, the following purification procedure was performed. First, 5 mL of NSY medium was inoculated with the cells from the agar slant and gently shaken at 28 °C until the stationary phase. After dilution, the cells were plated on NSY agar plate and grown at 28 °C for approximately 4 days. A single colony on the plate was used to inoculate the next 5 mL medium. This purification cycle was performed in triplicate. The new liquid culture started from a single colony on the third plate was mixed with glycerol (15% v/v) and stored at −80 °C. Careful tests in three labs confirmed the purity of the culture. The strain was deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany, under the number DSM 103376. The established glycerol stock was used as the seed culture for the following experiments.

For genomic DNA extraction, the cells were grown in 5 mL medium until the stationary phase. The cells were harvested by centrifugation and pretreated with lysozyme to lyse the cell walls. Then, the genomic DNA was extracted using a QIAGEN DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and used for the sequence analysis. The genomic DNA library was constructed using the tagmentation method (Nextera XT, Illumina, San Diego, CA, USA). The obtained library with an average insert size of approximately 1 kbp was selected using AMPure XP beads and sequenced using a MiSeq v3 600PE kit (Illumina). Approximately 5.58 million reads were obtained and assembled using the SPAdes 3.1 software. A single contig of 1.42 Mbp was obtained, and the genome sequence of the strain could be closed. The encoded genes were annotated using the RAST server [28]. The RpActR gene and the complete genome sequence were deposited at DDBJ/EMBL/GenBank under accession numbers LC144836 and CP015208, respectively.

2.2. Proton-pumping activity and ATP content in actinobacteria

Ca. R. planktonica were grown with or without 1 mM nicotine. The cells in glycerol stock were transferred to a tube containing 3 mL liquid medium and shaken at 25 °C for 3 days, then transferred to a 300-mL flask containing 40 mL medium. After 3 days, the culture reached the late exponential phase. The cells were then harvested at 3600 ×g for 5 min at 4 °C and washed twice in basal solution (0.3 g/L NaCl, 0.1 g/L $MgSO_4$) containing no buffering agent. The cells were suspended again in the basal solution and used for the following measurements.

The H^+-pump activity of RpActR was measured at 25 °C using a conventional pH electrode method that detects the light-induced acidification of the cell suspension due to the H^+-pump activity itself. The cell suspension was diluted with basal solution at an A_{660} of 0.5. The pH of this suspension was about 6.5–7.0. For the activation of RpActR, the cell suspension was irradiated with green LED light at 530 ± 17.5 nm (LXHL-LM5C, Philips Lumileds Lighting Co., San Jose, CA, USA). The H^+ transport was examined in the absence and presence of 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The resulting pH changes were converted to the molar amount of transported H^+ by adding known amounts of HCl after each measurement.

To assay the ATP content, the cell suspension was gently shaken overnight at 25 °C. Then, the cells were washed twice and finally suspended at an A_{660} of 0.1 in 10 mM Tris-AcOH, pH 7.8. This cell suspension was kept in the dark until the ATP assay. The ATP inside the cell was extracted using the Kinsiro ATP extraction reagent kit (LL-100-2; Toyo Ink, Tokyo, Japan), and the ATP level was determined using the Kinsiro ATP luminescence kit (LL-100-1; Toyo Ink) according to the manufacturer's instructions. Briefly, 5 μL of the cell suspension was mixed with the same volume of ATP extraction reagent and incubated for 20 s. To examine the effect of RpActR activity, the cell suspension was irradiated for 5 min with green LED light immediately prior to the ATP extraction. After the incubation, the resultant cell lysate was mixed with 100 μL of luciferin/luciferase reaction reagent. Immediately after mixing, the luminescence intensity at 560 nm was integrated for 20 s using a Model AB-2200 luminometer (ATTO, Tokyo, Japan). All procedures except LED irradiation were performed under dim conditions. The integrated luminescence value was converted to the ATP concentration using a standard line, which was constructed from the independent experiments. The total protein concentrations of the original cell