Biochimica et Biophysica Acta 1850 (2015) 1694–1703

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Mistic-fused expression of algal rhodopsins in *Escherichia coli* and its photochemical properties

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ARTICLE INFO

Article history:
Received 25 November 2014
Received in revised form 10 March 2015
Accepted 3 April 2015
Available online 11 April 2015

Keywords:
Rhodopsin
Bacteriorhodopsin
Algal rhodopsin
Proton transfer
Ion channel

ABSTRACT

Background: Since algal rhodopsins, the eukaryotic seven-transmembrane proteins, are generally difficult to express in *Escherichia coli*, eukaryotic cells have been used for heterologous expression. Mistic, a membrane-associated protein that was originally discovered in *Bacillus subtilis*, has been shown to improve the expression levels of many foreign integral membrane proteins in *E. coli* when used as a fusion partner linked to the N-terminus of cargo proteins.
Methods: Here, we expressed two algal rhodopsins with N- and C-terminal Mistic domains in *E. coli–Acetabularia* rhodopsin I (ARI) and *Chlamydomonas* sensory rhodopsin B (CSRB, channel rhodopsin 2). UV/VIS spectroscopy, pH titration of proton acceptor residue, laser-induced photolysis and electrophysiological measurement were used for investigating important residues in proton transport and spectroscopic characters of the proteins.
Results: Protein yield of two algal rhodopsins was enhanced, obtaining 0.12 mg of Mistic-ARI and 0.04 mg of Mistic-CSRB per liter of culture. Spheroplast expression Mistic-ARI had outward proton-pumping activity, indicating protein functionality. Asp89 of ARI changed its protonation state by light absorption, and Asp100 was important for O^{600} formation. Electrophysiology revealed that both residues took part in proton transport. The spectroscopic analyses of Mistic-CSRB revealed its characteristics.
Conclusions: Fusion to the membrane-integrating protein Mistic can enhance overexpression of eukaryotic type I rhodopsins in *E. coli*.
General Significance: These findings indicate that Mistic fusion and *E. coli* expression method could be an effective, low cost technique for studying eukaryotic membrane proteins. This may have useful implications, for example, in studying structural characteristics and optogenetics for rhodopsins.

1. Introduction

Type 1 microbial rhodopsins are light-driven ion pumps, channels, or light-activated signal transducers. They are composed of seven transmembrane helices and all-*trans* retinal or 13-*cis* retinal as a chromophore. The first identified rhodopsin, bacteriorhodopsin (BR), was found in *Halobacterium salinarum* (Archaea); *H. salinarum* contains three other rhodopsins: halorhodopsin (HR), sensory rhodopsin I (SRI), and sensory rhodopsin II (SRII). Genomic studies have also revealed a broad distribution of rhodopsins in microorganisms among the Eubacteria and Eukarya domains over the past decade.

Rhodopsin is also discovered from eukaryotic marine algae. Schilde reported in 1968, a fast, light-induced response of the transmembrane voltage from the giant unicellular marine alga *Acetabularia acetabulum* and suggested that rhodopsin might be the photoreceptor related to the response [1]. In 2004, Mandoli and co-workers investigated a cDNA sequence from juvenile *A. acetabulum* as a fragment of a tentatively opsin-encoding gene (Acetabulariaopsin, aop) [2]. Later, Hegemann and co-workers cloned a full-length opsin-cDNA from *A. acetabulum* and was named *Acetabularia* rhodopsin, abbreviated as AR. As expressing full-length version (AR_{1-279}; amino acids 1 to 279) and its truncated version (AR_{1-235}; amino acids 1 to 235) in *Xenopus* oocyte [3], they showed similar photoinduced outward currents through the oocyte membrane in the presence of various ion species and concluded that AR is the first light-driven proton-pumping rhodopsin found in a photosynthetic eukaryote. Also, they revealed that the intracellular H^+ donor group has a pK_a of 7.2, whereas the extracellular H^+ release group resembles a $pK_a > 9$.

At the same time, Kamo and co-workers including our group also obtained possible two clones from *A. acetabulum*. One gene named *Acetabularia* rhodopsin I, abbreviated as ARI_{1-237} (Database ID: HM070407) has revealed that it had almost same sequence compared with AR_{1-235} except one amino acid difference in 212th residue (Ilu for the ARI and Val for the AR). The photoelectric current of ARI in oocytes was analyzed [4]. Another gene found by our group was similar to AR but had clear differences, which was named *Acetabularia* rhodopsin II, abbreviated as ARII (Database ID: HM070408) [5]. We obtained

Abbreviations: CSRB, *Chlamydomonas* sensory rhodopsin B; ChR2, channel rhodopsin 2; ARI, *Acetabularia* rhodopsin I

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http://dx.doi.org/10.1016/j.bbagen.2015.04.002
0304-4165/

enough ARII with a cell-free expression system to allow them to examine this protein's photochemistry and X-ray crystal structure [5,6] and it was revealed that ARII was a light-driven proton pump [6].

Rhodopsin has also been found and characterized from *Chlamydomonas reinhardtii* and many other freshwater algae that are eukaryotic photosynthetic microorganisms. The earliest studies in *C. reinhardtii* described the positive phototactic reactions, the oriented swimming of cells along the direction of a light beam, in white light and the photophobic response, a reorientation of swimming direction induced by an abrupt increase in light intensity [7,8]. The first report of fast photoreceptor electric potential in the phototaxis was in the alga *Haematococcus pluvialis* [9]. Later, in *C. reinhardtii* including *Volvox carteri* also displayed extremely fast photoreceptor currents, carried by Ca^{2+} and H^+ under most conditions [10,11], through the plasma membrane of their eye after light excitation [11–13]. Some time later, one of the authors of the present paper demonstrated that two retinylidene proteins, *C. reinhardtii* sensory rhodopsin A (CSRA or ChR1 [channel rhodopsin 1]) and *C. reinhardtii* sensory rhodopsin B (CSRB or ChR2 [channel rhodopsin 2]), mediate phototaxis signaling in *C. reinhardtii* by *in vivo* analysis of photoelectrophysical analysis and motility responses in transformants with RNA interference (RNA*i*) [14,15]. In addition, studies in *Xenopus* oocytes and HEK293 cells revealed that ChR1 is selective for H^+, whereas ChR2 conducts monovalent ion such as Na^+, K^+ and even divalent cations such as Ca^{2+}, even though it mainly conducts H^+ [16–18]. Indeed, ChR2 act as an outwardly driven H^+ pump when it is reconstituted on planar lipid membranes or expressed in giant electrofused HEK293 cells, meaning the bifunctional character of the protein [19].

Because of the inward current character of the ChR2 cation channel under physiological conditions, exposure to blue light (470 nm) allows cations to enter the cell and causes a depolarization of neural cells [20–22]; as such, it has been used as an optogenetic tool to mediate action potential firing in neuronal tissue [23]. The crystal structure of channel rhodopsin was revealed by expression of its chimeric and truncated sequences, called C1C2, in insect cells [24]. Since these algal rhodopsins are difficult to express in *Escherichia coli*, eukaryotic cells, such as the yeast *Pichia pastoris*, insect cells, and mammalian COS-1 cells, have been used for heterologous expression, purification, and characterization [24–28].

In the present study, we expressed ARI and CSRB in *E. coli*, which is a less-expensive expression host than eukaryotic cells. We fused Mistic protein to ARI and CSRB and successfully purified as a fusion form. Mistic (an acronym for "membrane-integrating sequence for translation of integral membrane protein constructs") is a membrane-associated protein that was originally discovered in *Bacillus subtilis*. It has been shown to improve the expression levels of many foreign integral membrane proteins in *E. coli* membrane when used as a fusion partner linked to the N-terminus of cargo proteins [29–31]. Mistic part could be removed from ARI, but not from CSRB, so that we examined the photochemistry of the sole ARI protein and Mistic-CSRB.

As Asp89 and Asp100 residues of ARI are thought to be a proton acceptor and donor, respectively, we also constructed four mutants: D89N, D89E, D100N, and D100E. Three of those, D89E, D100N, and D100E, were successfully expressed, and their photochemical properties were measured.

2. Material and methods

2.1. Construction of Mistic cassette

To fuse various cargo proteins with Mistic (Database ID: AY874162), 839 bases of Mistic cassette were synthesized commercially (BMS, South Korea) (Fig. 1A). The multiple cloning site (MCS) which will be inserted by various rhodopsins and two Mistics containing a His_6 tag at the N-and C-termini for each are joined by the flexible linker ASASNGASA [32]. For Mistic cleavage from the rhodopsins, the protease cleavage site called Factor X Ile-Asp-Gly-Arg cleaved after arginine residue by Factor Xa protease. The Mistic cassette was inserted into the pKA001 vector with NdeI and NotI restriction enzyme sites. pKA001 is derived from pKJ900 [33]; the second NdeI site in the middle of the pKJ900 vector was eliminated so that the NdeI site could be used in cloning. The resulting pKA001 vector with the Mistic cassette was called pMisCa (Fig. 1A).

2.2. Heterologous expression and purification of ARI and CSRB in E. coli

We previously identified ARI gene (Database ID: AEF12206) from *Acetabularia* juvenile-specific cDNA by PCR [34] and used entire sequence for the ARI construct without any codon optimization. For the CSRB construct, truncated $CSRB_{1\text{-}300}$ (amino acids 1 to 300) was used out of total 717 amino acid residues of the CSRB (Database ID: AAM44042) [14]. The CSRB construct with *E. coli* codon optimization was synthesized commercially (BMS, South Korea). ARI and CSRB genes were inserted into pMisCa using SacII and XhoI restriction enzyme sites in the multiple cloning site (Fig. 1A). The Mistic fusion proteins of ARI and CSRB were expressed in *E. coli* β/UT and purified as previously described [35]. Then β/UT *E. coli* cell is produced by transforming plasmid pORANGE into *E. coli* UT5600 cells. The pMisCa transformants of β/UT cells could produce inserted gene with internal all-*trans* retinal from β-carotene, resulting in rhodopsin protein. The transformed cells were pre-cultured in 5 ml of Luria-Broth (LB) for 16 h with 50 μg/ml of ampicillin, transferred 1% of seed culture to 500 ml of LB medium, induced with 1 mM IPTG (Applichem, USA) and 0.2% (+)-L-arabinose (Sigma, USA) at 0.8 OD_{600}, and incubated further for 24 h at 30 °C. The collected cells were sonicated (Branson sonifier 250) for 10 min on ice and centrifuged (Appendorf Centrifuge 5810 R, Germany) again at 2500 ×*g*, 4 °C for 20 min for discarding unbroken cells. The soluble fraction was taken and ultracentrifuged (Beckman XL-90 ultracentrifuge, Rotor no. 70Ti, USA) again at 35,000 ×*g*, 4 °C for 1 h for isolating membrane fraction. The suspended pellet was treated with 1% n-dodecyl-β-D-maltopyranoside (DDM) (Anatrace, USA) for 1 h at 4 °C. The solubilized fraction was incubated with Ni^{2+}-NTA agarose (Qiagen, USA) and eluted with buffer (50 mM Tris–HCl (pH 7.0), 500 mM NaCl, 0.2% DDM and 250 mM imidazole (Sigma, USA)). The purified fusion proteins were solubilized in 0.2% DDM, 50 mM Tris–HCl (pH 7.0), and 500 mM NaCl. In order to remove Mistic parts from the fusion protein, especially here for Mistic-ARI, it was treated with Factor Xa protease for cleavage for 6 h at RT in 0.2% DDM, 50 mM Tris–HCl (pH 8.0), 500 mM NaCl and 2 mM $CaCl_2$, followed by purifying with Ni^{2+}-NTA agarose resin again to bind and remove the Mistic part. Finally, the flow through rhodopsin protein, ARI, was collected and solubilized in 0.2% DDM, 50 mM Tris–HCl (pH 7.0), and 500 mM NaCl.

2.3. Absorption spectroscopy and pK_a determination

Overall procedures were described previously [35]. Briefly, the pH-dependent absorption spectra of purified ARI and CSRB were recorded with a Shimadzu UV–visible (UV–Vis) spectrophotometer (UV-2550). The corrected ratio of protonated and deprotonated forms at different pH values was determined as previously described [36] from the intensities of the absorption bands that appear at λ_{max} of each new component as the pH is elevated. The data were fitted to the Henderson–Hasselbach equation in the form ($y = A/(1 + 10^{pH\text{-}pKa})$) using Origin Pro 6.1, where *A* represents the maximal amplitude of relative absorbance changes.

2.4. Light-induced static absorbance changes and flash photolysis spectroscopy

Light-induced static absorbance changes of ARI and CSRB were measured on Scinco S-3100 spectrometer (Scinco, South Korea). Sample