Journal of Structural Biology 184 (2013) 2–11

Contents lists available at SciVerse ScienceDirect

Journal of Structural Biology

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

Role of trimer–trimer interaction of bacteriorhodopsin studied by optical spectroscopy and high-speed atomic force microscopy

Hayato Yamashita [a,1,3], Keiichi Inoue [b,3], Mikihiro Shibata [a,2], Takayuki Uchihashi [a,c], Jun Sasaki [b], Hideki Kandori [b], Toshio Ando [a,c,*]

[a] Department of Physics, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan
[b] Department of Frontier Materials, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, Japan
[c] Bio-AFM Frontier Research Center, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan

ARTICLE INFO

Article history:
Available online 24 February 2013

Keywords:
High-speed atomic force microscopy
Bacteriorhodopsin
Trimer
Point mutation
Low-temperature UV–visible spectroscopy
Proton pumping efficiency

ABSTRACT

Bacteriorhodopsin (bR) trimers form a two-dimensional hexagonal lattice in the purple membrane of *Halobacterium salinarum*. However, the physiological significance of forming the lattice has long been elusive. Here, we study this issue by comparing properties of assembled and non-assembled bR trimers using directed mutagenesis, high-speed atomic force microscopy (HS-AFM), optical spectroscopy, and a proton pumping assay. First, we show that the bonds formed between W12 and F135 amino acid residues are responsible for trimer–trimer association that leads to lattice assembly; the lattice is completely disrupted in both W12I and F135I mutants. HS-AFM imaging reveals that both crystallized D96N and non-crystallized D96N/W12I mutants undergo a large conformational change (i.e., outward E–F loop displacement) upon light-activation. However, lattice disruption significantly reduces the rate of conformational change under continuous light illumination. Nevertheless, the quantum yield of M-state formation, measured by low-temperature UV–visible spectroscopy, and proton pumping efficiency are unaffected by lattice disruption. From these results, we conclude that trimer–trimer association plays essential roles in providing bound retinal with an appropriate environment to maintain its full photoreactivity and in maintaining the natural photo-reaction pathway.

1. Introduction

Intermolecular interactions play a pivotal role in the function of membrane proteins. Numerous membrane proteins interact with each other or other components in two-dimensional membrane environments, e.g., self-assembly into oligomers or crystals (Essen et al., 1998; Persike et al., 2001; van Huizen et al., 1999; Colom et al., 2012), receptor-mediated protein–protein interactions for signal transduction (Szidonya et al., 2008), and the membrane localization of proteins via association with lipid rafts (Simons and Ikonen, 1997). Thus, elucidating the intermolecular packing of membrane proteins is essential for understanding the structure–function relationship and the general principle of membrane protein assembly.

Bacteriorhodopsin (bR), a light-driven proton pump in *Halobacterium* (*H.*) *salinarum* (Oesterhelt and Stoeckenius, 1971), is one of the best-characterized transmembrane proteins (see reviews: Haupts et al., 1999; Lanyi, 2004; Lanyi, 2006; Hirai et al., 2009). Under the native condition, bR forms trimers that assemble into a two-dimensional hexagonal lattice called the purple membrane (PM) (Blaurock and Stoeckenius, 1971). Previously, numerous structural studies of bR in PM were performed under unphotolyzed (Sass et al., 2000) or frozen activated (Luecke et al., 1999; Subramaniam and Henderson, 2000; Vonck, 2000; Lanyi and Schobert, 2003; Hirai and Subramaniam, 2009) states. Recently, by means of high-speed atomic force microscopy (HS-AFM) (Ando et al., 2001; Ando et al., 2008), light-induced conformational changes of bR have been visualized in real-time and real-space (Shibata et al., 2010; Shibata et al., 2011). HS-AFM movies have revealed that the E–F inter-helical loop of each bR monomer moves outwards from its trimer center upon photo-excitation from the ground state to the M_N state, which results in contact among three nearest-neighbor bR monomers, each belonging to a different adjacent trimer. This triad of

Abbreviations: AFM, atomic force microscopy; bR, bacteriorhodopsin; PM, purple membrane; HPLC, high-performance liquid chromatography; HS-AFM, high-speed atomic force microscopy; WT, wild-type.

* Corresponding author at: Department of Physics, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan. Tel.: +81 76 264 5663; fax: +81 76 264 5739.
E-mail address: tando@staff.kanazawa-u.ac.jp (T. Ando).
[1] Present address: Department of Pharmacology, School of Medicine, Keio University, Tokyo, Japan.
[2] Present address: Department of Neurobiology, Duke University Medical Center, North Carolina, USA.
[3] These authors contributed equally to this work.

1047-8477/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jsb.2013.02.011

nearest-neighbor monomers is designated the 'trefoil'. This bR–bR interaction within a trefoil, which occurs under a relatively strong light illumination, engenders both positive and negative (i.e., bipolar) cooperative effects in decay kinetics; an early activated monomer within a trefoil decays more slowly, whereas the latest activated monomer within a trefoil decays faster than that in the case in which only one monomer within a trefoil is activated (and hence no bR–bR interaction occurs) under weak light illumination. However, because of bipolar cooperative effects, the overall turnover rate is maintained over a wide range of light intensities (Shibata et al., 2010).

An HS-AFM observation has also showed that a bR trimer occasionally rotates around a pivotal point at the interface between dense crystal and non-assembled areas of PM, suggesting the presence of specific inter-trimer interaction sites at the outer rim of a bR trimer (Yamashita et al., 2009). This is consistent with the result obtained from a neutron diffraction study suggesting that some of the aromatic residues (W10, W12, Y131, and F135) located vertically no farther than 5 Å into the extracellular side of the membrane and laterally at the outer rim of a trimer are involved in lattice formation (Weik et al., 1998). This suggestion is derived from the measured glycolipid distribution in the membrane and the possible hydrophobic stacking between aromatic side chains and carbohydrates (Qian et al., 1995) as well as from the close proximity between W10–W12 and Y131–F135 in the bR assembly. However, a more definite and specific identification of amino acids responsible for lattice formation has not been performed ever since.

In the present study, we specified amino acid residues involved in trimer–trimer association by preparing five bR mutants in which isoleucine was substituted for the aromatic residues mentioned above or phenylalanine was substituted for W12. HS-AFM observations of these mutants showed that the trimers of bR mutants (W12I and F135I) do not assemble and instead rapidly diffuse in the membrane. Furthermore, the HS-AFM observation of the D96N/W12I bR mutant revealed that lattice disruption reduces the rate of light-induced conformation change, which is proportional to light intensity. Interestingly, the spectroscopic and proton pumping assay studies, however, showed that proton pumping efficiency is unaffected by W12I mutation. Taking these results into consideration, we propose a model in which lattice disruption causes the photo-reaction pathway to branch into two: the original productive pathway and a new non-productive pathway. In the non-productive pathway, no significant structural change occurs in bR and no proton is pumped out; however, the kinetics on this pathway is much faster than that on the original pathway. This model quantitatively well explains all the results obtained in this study. Thus, trimer–trimer association plays essential roles in providing bound retinal with an appropriate environment to maintain its full photo-reactivity and in maintaining the natural photo-reaction pathway.

2. Materials and methods

2.1. Plasmid constructs and site-directed mutagenesis

A template plasmid containing the bop gene (1.6 kbp) cloned into the vector pGEM-T easy (Promega) was used (Shibata et al., 2007). Point mutations were introduced into the bop gene by PCR mutagenesis (Stratagene) as follows; W10I, Trp10 → Ile; W12I, Trp12 → Ile; Y131I, Tyr131 → Ile; F135I, Phe135 → Ile; W12F, Trp12 → Phe; D96N, Asp96 → Asn; and D96N/W12I, Asp96 → Asn and Trp12 → Ile. The mutated fragments were transferred to the vector pMPK69 for transformation (Peck et al., 2000), which contains a gene conferring resistance to the 3-hydroxy-3-methylglutaryl CoA reductase inhibitor (simvastatin, Wako) on *H. salinarum*. The resultant mutations were confirmed by sequencing the plasmids prior to the transformation of *H. salinarum*.

2.2. Transformation of H. salinarum and purification of bacteriorhodopsin mutants

H. salinarum strain MPK409 (Peck et al., 2000) was transformed using the plasmids described above. The transformation procedure was essentially as described previously (Cline and Doolittle, 1987). Briefly, 10 mL of cells in the early logarithmic growth phase was collected by centrifugation (6000*g*, 10 min) and resuspended in 500 μL of a spheroplasting solution to facilitate plasmid DNA transfection into the cells. 200 μL-aliquots of the suspension were added to 10 μL of 0.5 M EDTA in a spheroplasting solution and gently mixed. After 20 min, 15 μL of a plasmid DNA solution was added, and the resulting solution was incubated for 5 min. To this solution, an equal volume (225 μL in this case) of 60% polyethylene glycol 600 in a spheroplasting solution was added; the solution obtained was mixed gently by back and forth tilting of the tube. After 20 min of additional incubation, 10 mL of a regeneration salt solution containing 15% sucrose was added. Cells were pelleted by microcentrifugation at 5000 rpm for 20 min, and 10 mL of a regeneration salt solution was again added to the resultant pellet. After incubation for 24 h at 37 °C, 100-μL samples were spread on agar plates containing 15% sucrose and 10 μg/mL simvastatin. After 10 days of incubation at 37 °C, purple colonies were picked and grown in a complex culture medium with 10 μg/mL simvastatin. After incubation for 24 h at 37 °C, 100-μL samples were spread on agar plates containing 250 μg/mL 5-fluoroorotic acid (Wako). After a week of incubation, purple colonies were picked and grown again in a complex culture medium containing 50 μg/mL uracil, as uracil cannot be synthesized by the transformed cells lacking the *URA3* gene coding for orotine-5-monophosphate decarboxylase that converts 5-fluoroorotic acid into toxic 5-fluorouracil. The purple membranes were isolated and purified by a usual method (Oesterhelt and Stoeckenius, 1974). As W12I, F135I and D96N/W12I do not form 2D crystals in the native membrane, the bottom layer containing the highest bR content was collected by sucrose density gradient centrifugation. After removing sucrose by sedimentation, the pellets were suspended in a solution (Buffer-A) containing 10 mM phosphate (pH 7.0) and 300 mM KCl. For AFM observation, the samples were diluted to ~0.1 mg/mL in Buffer-A.

2.3. HS-AFM observation

The laboratory-built HS-AFM apparatus used in this study is similar to that previously reported (Ando et al., 2008). The detailed procedures towards HS-AFM imaging are reported elsewhere (Uchihashi et al., 2012). HS-AFM images were acquired in the tapping mode. To detect cantilever deflection, we used an optical beam deflection detector equipped with an infrared laser (980 nm) to avoid exciting bR. The laser beam was focused onto a small cantilever using a ×50 objective lens. The cantilever with a spring constant of ~0.2 N m^{-1} (Olympus) is 6–7 μm long, 2 μm wide and 90 nm thick. Its resonant frequency and quality factor in an aqueous solution are ~1 MHz and ~2, respectively. An amorphous carbon tip was grown on the original cantilever tip by electron beam deposition. Tip length was adjusted to ~1 μm. The tip apex was sharpened by plasma etching under argon gas (~4 nm in radius). For HS-AFM imaging, the cantilever free-oscillation amplitude was set at ~1 nm, and the set-point amplitude was set at 90% of the free-oscillation amplitude. A sample stage made of quartz glass was placed on the *z*-scanner, and a 1.5-mm-diameter mica disk was glued onto the sample stage. A 2-μL sample droplet