Real-Time Atomic Force Microscopy

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Structural Changes in Bacteriorhodopsin in Response to Alternate Illumination Observed by High-Speed Atomic Force Microscopy**

*Mikihiro Shibata, Takayuki Uchihashi, Hayato Yamashita, Hideki Kandori, and Toshio Ando**

The membrane protein bacteriorhodopsin (bR), found in *Halobacterium salinarum*, functions as a light-driven proton pump transferring protons across the membrane from the cytoplasmic side to the extracellular side.[1] bR is comprised of seven transmembrane α helices (named A–G) surrounding the retinal chromophore covalently bound to Lys216 through a protonated Schiff base.[2] bR assembles into trimers, which are packed into two-dimensional hexagonal lattices, the so-called purple membrane.[3] Upon absorption of light, photoisomerization from the all-*trans* to the 13-*cis* conformation of retinal takes place, with subsequent primary proton transfer from the Schiff base to Asp85, which triggers a cascade of changes in bR structure. A series of intermediates designated J, K, L, M, N, and O have been defined by spectroscopy, and M (M_{410}, having a light absorbance peak at 410 nm) is the only intermediate containing a deprotonated Schiff base.[1]

The light-induced conformational changes in bR have been studied by various methods.[4–8] Particularly important are crystallographic structures in the frozen activated state of the wild type (WT) and of bR mutants, which were solved at atomic resolution.[6] The common understanding regarding the bR structure during the photocycle is that the proton channel on the cytoplasmic surface is opened by the tilting of helix F away from the protein center.[6] This alteration is followed by rearrangement of the interhelix E–F loop, thus resulting in large-scale conformational changes in the M and N intermediates.[5,7,8]

Atomic force microscopy (AFM), which can visualize nanometer-scale objects in various environments,[9] has also been used for structural studies of bR under aqueous conditions, and high-resolution images that allow the identification of individual interhelix loops are obtained.[10,11] To date, conventional AFM has provided structural information not only of bR but also of other membrane proteins.[10–12] However, conventional AFM can only show static or slow time-lapse images of biomolecules because of its poor temporal resolution. Over the last decade, however, the scan speed of AFM has been increased by various efforts.[13–15] Recent advances in fast scanning techniques of AFM have demonstrated that it is possible to observe dynamic behavior of single protein molecules in action.[16]

Using high-speed atomic force microscopy (HS-AFM), we recently succeeded in the real-space and real-time observation of light-induced conformational changes of bR under physiological conditions.[17] The photocycle of the wild type at neutral pH values proceeds very quickly (ca. 10 ms), and hence, the conformational changes cannot be clearly imaged even by our HS-AFM. To slow down the photocycle, we used the D96N bR mutant, which has a longer photocycle (ca. 10 s at pH 7) but still retains a proton pumping ability.[18] Reversible dynamic structural changes in bR in response to green light appeared in the molecular movies.[17]

It is known that the all-*trans* to 13-*cis* isomerization of retinal can be reversed by subsequent illumination with light of a different wavelength.[19] After M_{410} is formed upon green illumination, it can be driven back to the ground state (bR_{570}) by blue light. However, the spectroscopically detected reversal does not necessarily indicate the reversal of the entire protein structure. Namely, it is not certain to date whether the protein structure is tightly coupled to the conformation of retinal. Herein, we address this issue by directly visualizing D96N bR under alternate illumination with green ($\lambda = 532$ nm) and blue ($\lambda = 408$ nm) light using HS-AFM.

Figure 1 presents AFM images of the D96N bR mutant at the cytoplasmic surface captured at 1 frame s^{-1} (see Movies S1 and S2 in the Supporting Information). Under the green light, a part of each bR molecule is displaced counterclockwise and outward from the trimer center (compare images at 15 and 28 s in Figure 1), as we reported.[17] As a result, three nearest-neighbor bR monomers, each of which belongs to a different adjacent trimer, are brought into contact with each other. Figure 2 shows the displacement of the centers of mass as a function of time for three different bR monomers (M1–M3 in Figure 2). Note that each "center of mass" was calculated from the height distribution at a surface region of each bR monomer in the AFM image (see the Supporting Information for details). Under these illumination conditions, once bR molecules change their conformation by green light, the conformation of most bR molecules appears to remain in the activated state even after the light is turned

[*] Dr. M. Shibata, Prof. Dr. T. Uchihashi, Dr. H. Yamashita, Prof. Dr. T. Ando
Department of Physics, Kanazawa University
Kakuma-machi, Kanazawa (Japan)
Fax: (+81) 76-264-5739
E-mail: tando@kenroku.kanazawa-u.ac.jp
Homepage: http://www.s.kanazawa-u.ac.jp/phys/biophys/index.htm
Prof. Dr. H. Kandori
Department of Frontier Materials, Nagoya Institute of Technology
Showa-ku, Nagoya (Japan)

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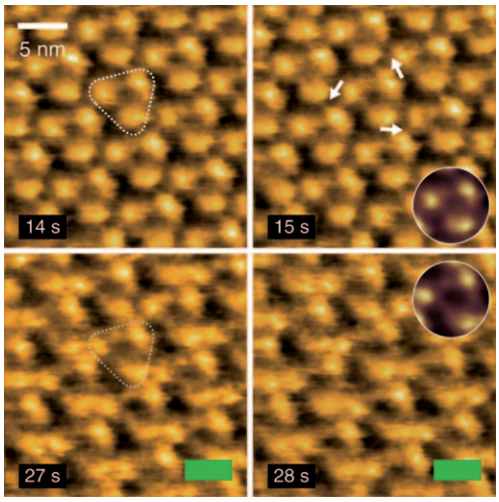

Figure 1. HS-AFM images of the D96N bR mutant at the cytoplasmic surface. 1 frame s^{-1}, 200×200 pixels. The membranes were adsorbed onto a mica surface in 10 mM phosphate (pH 8) and 300 mM KCl. A bR trimer is highlighted by the white triangles. The white arrows indicate the direction of the conformational change in bR (15 s). The green bars indicate application of 532 nm green light (27 and 28 s). The insets in the images at 15 and 28 s are averaged images of a bR trimer captured in the dark and under green light, respectively.

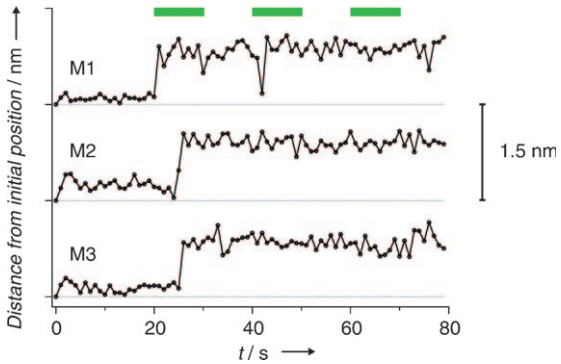

Figure 2. Displacement of centers of mass for three D96N bR molecules measured as a function of time at pH 8 on the cytoplasmic side. The average displacement induced by light is (0.80 ± 0.13) nm (mean ± standard deviation, s.d.). The green bars show the periods of green illumination.

off. The molecules behave in this way because the lifetime of the activated state of D96N bR at pH 8 is longer than the duration of the on–off cycle of illumination.

The protruding parts of bR around helices E and F displace outward from the trimer center under illumination. In addition, a previous static AFM imaging study of bR assigned the prominent protrusion in the AFM topographs of bR to the cytoplasmic E–F loop.[10] Thus, we conclude that the observed conformational change of bR originates from the displacement of the E–F loop. There have been a number of reports on the displacement of helices on the cytoplasmic side of bR detected by X-ray and electron diffraction techniques.[6] However, the diffraction techniques are generally unable to determine the displacement of loop regions because of the disordered structures. In contrast, electron paramagnetic resonance (EPR) spectroscopy can detect the E–F loop displacement as a change in the distance between a pair of nitroxide spin labels attached to the E–F loop and to another locus on the cytoplasmic surface. Thorgeirsson et al. reported that upon photoactivation, the distance between 103C (in the C–D loop) and 163C (in the E–F loop) of the A103C/M163C double mutant and the distance between 35C (in the A–B loop) and 163C of the S35C/M163C double mutant increase by 0.65 and 0.4 nm, respectively.[5] From various interspin-distance measurements, Xiao et al. suggested that the E–F loop is displaced counterclockwise and outward from the protein center.[7] These studies suggest that the E–F loop is displaced by a larger distance than the helices move. Our observation of the counterclockwise rotation and displacement of the EF loop by approximately 0.8 nm is consistent with these EPR studies.

Figure 3 shows images of the cytoplasmic surface under alternate illumination with green and blue light. In contrast to the case of only green light, the analysis of the center of mass of each molecule clearly shows that bR molecules undergo alternate conformational changes, which are mostly synchron-

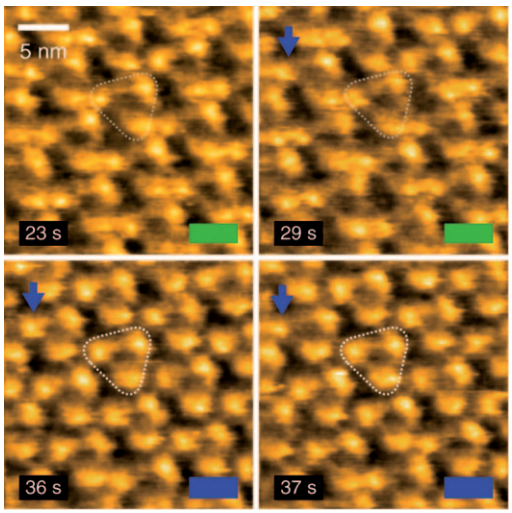

Figure 3. HS-AFM images of D96N bR mutant at the cytoplasmic surface illuminated by green and blue light. The green and blue bars indicate application of 532 nm green light (23 and 29 s) and 408 nm blue light (36 and 37 s). Blue arrows highlight an instance of bR activation by blue light.

ized with the alternate application of green and blue light (Figure 4; see also Movies S3 and S4 in the Supporting Information). The blue-light-induced conformational change back to the ground state is caused by the photo-back-reaction, not by the normal turnover of the photocycle. Because bR in the ground state also absorbs blue light, deactivated bR is sometimes reactivated under the blue light (blue arrows at 36 and 37 s in Figure 3, Movie S3 in the Supporting Information, and blue arrows in Figure 4). These observations clearly show that the conformation of bR is tightly coupled to the conformation of retinal.

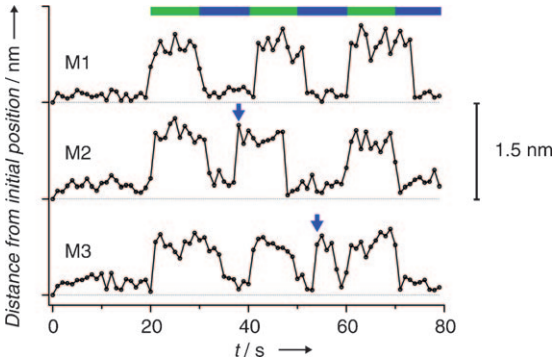

Figure 4. Displacement of centers of mass for three D96N bR molecules measured at pH 8 on the cytoplasmic side. The green and blue bars show periods of green and blue light application, respectively. Blue arrows indicate bR activation by blue light.

Next, we examined whether the activated state of bR observed by AFM contains not only M_{410} but also other intermediates that are spectroscopically different from M_{410}. To address this issue, the photocycle was initiated by brief green-light illumination, and then blue light was briefly applied with different delay times. Figure 5a shows the decay of the activated state after the green light was applied for 3 s. Monomers whose centers of mass are displaced outward by approximately 0.8 nm are counted as being in the activated state. After the green light is switched off, bR molecules thermally return to the ground state through the photocycle (Figure 5a), with a time constant of about 40 s. In contrast, Figure 5b shows the decay of the activated state induced by blue light applied at a delay time of 30 s after turning off the green light. Although a few bR molecules are deactivated and then reactivated during the blue-light illumination, these molecules are not counted in this analysis. Before the blue light is turned on, the fraction of activated molecules gradually decreases in the normal course of the photocycle, but it is abruptly decreased nearly to zero after the brief blue-light illumination (Figure 5b and Figure S1 in the Supporting Information). The time constant for the decay during the blue-light illumination is about 1 s, independent of the delay time between the two illumination periods (Table S1 in the Supporting Information). Thus, we conclude that the conformation of D96N bR in the activated state observed by AFM solely corresponds to the M_{410} intermediate, which efficiently absorbs blue light.

In conclusion, the AFM observations presented herein demonstrate alternate conformational changes in bR responding to alternate illumination with light of different wavelengths. The molecular movies directly reveal that the conformation of bR is tightly governed by the conformation of retinal without a time delay, at least within the time resolution of the study (1 frame s^{-1}). As demonstrated herein, direct and dynamic observation of functioning protein molecules is a powerful new approach to study conformational changes in proteins induced by external stimuli.

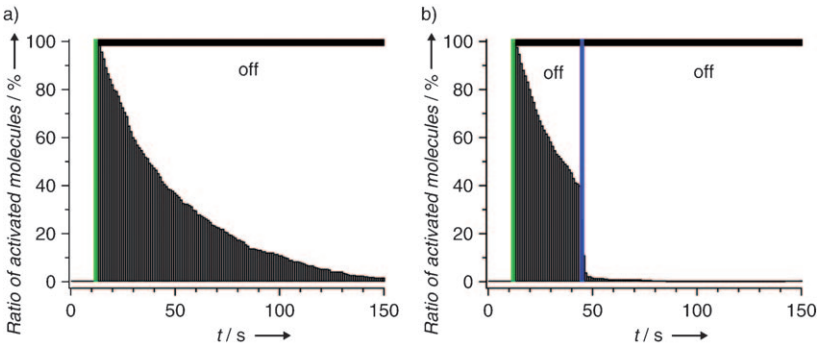

Figure 5. Decay of the activated state of D96N bR detected by HS-AFM at pH 8. Green and blue regions indicate application of green and blue light, respectively. Illumination periods of both green and blue light are 3 s. a) Decay through the normal course of the photocycle with a time constant of (39 ± 0.27) s (mean ± s.d., the total number of analyzed bR molecules $n = 354$). b) Decay induced by blue light ($n = 389$).

Experimental Section

Sample preparation: Purple membranes containing the D96N bR mutant were isolated from *Halobacterium salinarum* as described.[17,20] The samples were suspended in a solution containing 10 mM phosphate buffer (pH 8) and 300 mM KCl.

HS-AFM: The experimental conditions of the high-speed AFM measurements were similar to those reported previously.[17] The AFM images were obtained in the tapping mode for the single-layered purple membranes on a mica surface under solution at room temperature. To detect the cantilever deflection, we used an optical beam deflection detector equipped with an infrared laser (980 nm). The laser beam was focused onto a small cantilever using a × 50 objective lens. The cantilevers (Olympus) are 6–7 μm long, 2 μm wide, and 90 nm thick with a spring constant of 0.1–0.2 $N m^{-1}$. Their resonant frequency and quality factor in an aqueous solution are approximately 1 MHz and 2, respectively. An amorphous carbon tip was grown on the original tip by electron beam deposition. The tip length was adjusted to approximately 1 μm, and the tip apex was sharpened by plasma etching under argon gas (ca. 4 nm radius). The bR sample was irradiated with green (532 nm) or blue (408 nm) laser light (both ca. 0.5 μW) through the × 50 objective lens.

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