Structural changes in bacteriorhodopsin in response to alternate illumination observed by high-speed atomic force microscopy

Real-Time AFM Imaging

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

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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 via 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 all-*trans* to 13-*cis* conformation of the retinal takes place, followed by the primary proton transfer from the Schiff base to Asp85 that triggers a cascade of changes in the bR structure. A series of intermediates designated J, K, L, M, N, and O are defined by spectroscopy, and M (M_{410}) 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] and particularly crystallographic structures in the frozen activated state of the wild type (WT) and bR mutants were solved at the 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, 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 faithful high-resolution images capable of distinguishing the individual interhelix loops are obtained.[10, 11] To date, conventional AFM have 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 proceeds very fast (~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 that has a longer photocycle (~10 s at pH 7) but still retains a proton pumping ability.[18] Responding to green light, reversible dynamic structural changes in bR appeared in the molecular movies.[17]

It is known that the all-*trans* to 13-*cis* isomerization of retinal can be reversed by second illumination with 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 yet certain whether the protein structure is tightly coupled to the conformation of retinal. Here, we address this issue by directly visualizing D96N bR under alternate illumination of green (λ= 532 nm) and blue (λ= 408 nm) light, using HS-AFM.

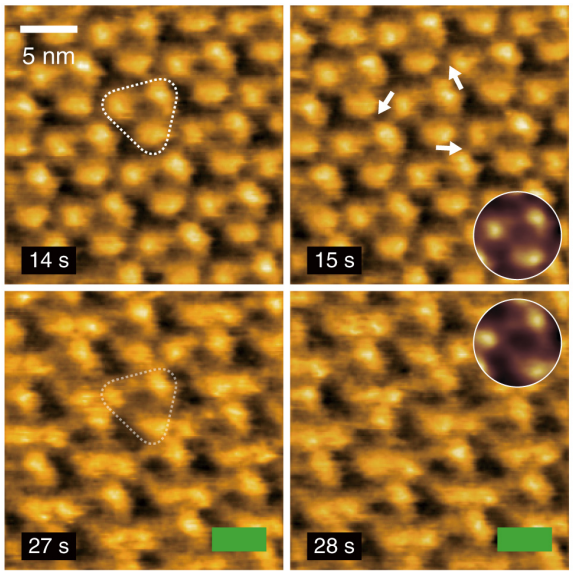

Figure 1. HS-AFM images of D96N bR mutant at cytoplasmic surface. Frame rate, 1 frame/s; number of pixels, 200 × 200. The membranes were adsorbed onto a mica surface in 10 mM phosphate (pH 8) and 300 mM KCl. A bR trimer is highlighted by the white triangles. The white arrows in a counterclockwise rotation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

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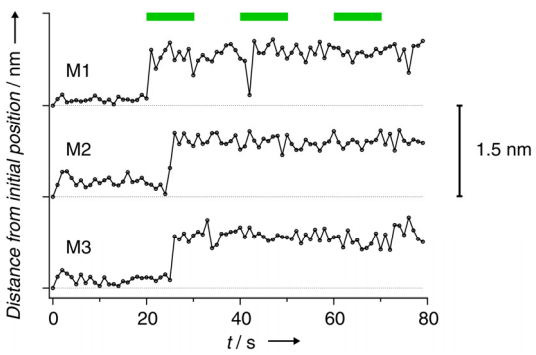

Figure 2. Displacement of centers of mass for three D96N bR molecules measured as a function of time at pH 8 on the cytoplasmic side. The average displacement induced by light is 0.80 ± 0.13 (mean ± s.d.) nm. The green bars show the periods of green illumination.

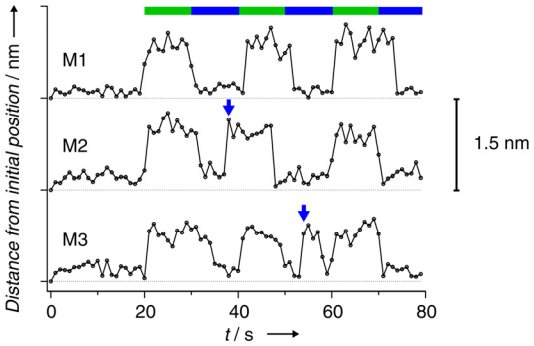

Figure 4. Displacement of centers of mass for three D96N bR molecules measured at pH 8 on the cytoplasmic side. The green and blue bars show periods of green and blue light application, respectively. Blue arrows indicate events of bR molecule activation by blue light.

Figure 1 presents AFM images of D96N bR mutant at the cytoplasmic surface captured at 1 frame/s (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 s and 28 s in Figure 1), as we reported previously.[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 (Method in the Supporting Information). Under this illumination condition, 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 off. This is 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 used.

The protruding parts around helices E and F displace outward from the trimer center under illumination. In addition, a previous static AFM imaging study of bR has assigned the prominent protrusion in the AFM topographs of bR to the cytoplasmic E-F loop.[10] Thus, we conclud that the observed conformational change of bR is originated 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 another locus on the cytoplasmic surface. Thorgeirsson *et al*. reported that upon photo-activation, 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 become larger by 0.65 nm 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 movement of helices. Our observaion of the counterclockwise rotation and displacement by ~0.8 nm of the EF loop is consistent with these EPR studies.

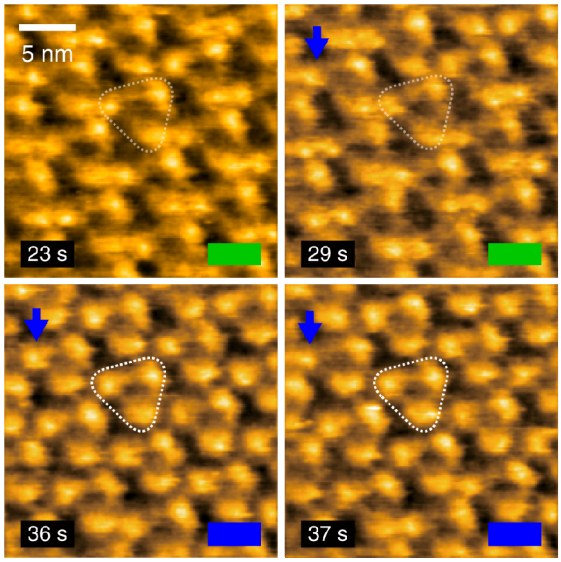

Figure 3. HS-AFM images of D96N bR mutant at cytoplasmic surface illuminated by green and blue lights. The green and blue bars indicate application of 532-nm green light (images at 23 s and 29 s) and 408-nm blue light (images at 36 s and 37 s), respectively.

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 synchronized with the alternate green/blue light application (Figure 4; see 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 turn-over 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 s 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.

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. Here, monomers whose centers of mass are displaced outward by ~0.8 nm are counted as those 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

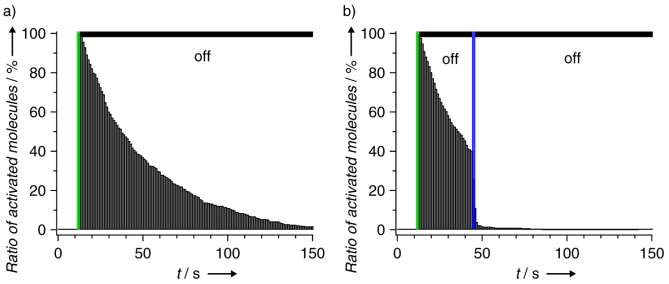

Figure 5. Decay of the activated state of D96N bR detected by HS-AFM at pH 8. Green and blue regions indicate application of green and blue light, respectively. Illumination periods of both green and blue light are 3 s. (a) Decay through the normal course of photocycle with a time constant of 39 ± 0.27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

is gradually decreasing 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 here demonstrate alternate conformational changes in bR responding to alternate illumination. 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 used here (1 frame/s). As demonstrated in the present study, direct and dynamic observation of functioning protein molecules is a powerful new approach to studying conformational changes in proteins induced by external stimuli.

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) used are 6–7 μm long, 2 μm wide, and 90 nm thick with a spring constant of 0.1–0.2 N/m. Their resonant frequency and quality factor in an aqueous solution are ~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 ~1 μm, and the tip apex was sharpened by plasma etching under argon gas (~4 nm in radius). For illumination of the bR sample, ~0.5 μW green laser (532 nm) or blue laser (408 nm) light was irradiated through the ×50 objective lens.

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Real-Time AFM Imaging

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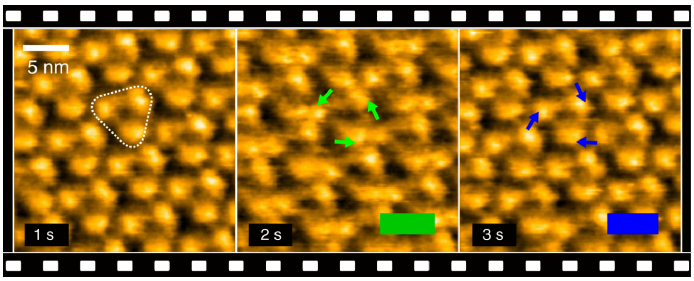

Using high-speed atomic force microscopy, we visualized light-induced structural changes of the D96N bacteriorhodopsin mutant under alternate two-color illumination. Under green light illumination, each bR is displaced outward from the trimer center. This activated structure is immediately driven back to the ground state by the subsequent blue light illumination. Thus, the conformation of bR is tightly coupled to the conformation of retinal.