A molecular movie of structural changes in the light-driven proton pump bacteriorhodopsin

Bacteriorhodopsin (bR) is a light-driven proton pump derived from *Halobacterium salinarum*. The protein harvests the energy content of light to drive conformational changes leading to unidirectional proton transport (Fig. 1(a)). The resulting proton concentration gradient is converted by adenosine triphosphate (ATP) synthase into ATP or is coupled to other transport processes.

bR comprises a seven-transmembrane helix and contains a buried all-*trans* retinal chromophore that is covalently bound to Lys216 (Fig. 1). The all-*trans* retinal undergoes isomerization to the 13-*cis* configuration by the absorption of light, which initiates a photocycle, accompanying a sequence of spectral and structural changes.

The primary proton transfer occurs between a protonated Schiff base and Asp85. In the resting-state bR structure, the protonated Schiff base and Asp85 form hydrogen-bond interactions with three water molecules and two amino acid residues as shown in Fig. 2(a). The hydrogen-bond network results in a difference of 11 orders of magnitude between the proton affinities of the primary donor and acceptor, which prevents the leakage of protons from the extracellular medium to the cytoplasm. After light excitation, these proton affinities approach each other to facilitate spontaneous proton exchange, but this raises the question of what brings the change of the proton affinities. It is also puzzling why it takes microseconds for the primary proton transfer to occur when the Schiff base and Asp85 are initially separated by only 4 Å and a water-mediated proton exchange pathway between the proton donor and acceptor is seen in the resting state. Moreover, protons are pumped from the cytoplasm to the extracellular side, yet retinal isomerization redirects the Schiff base proton away from the extracellular side and toward the cytoplasm.

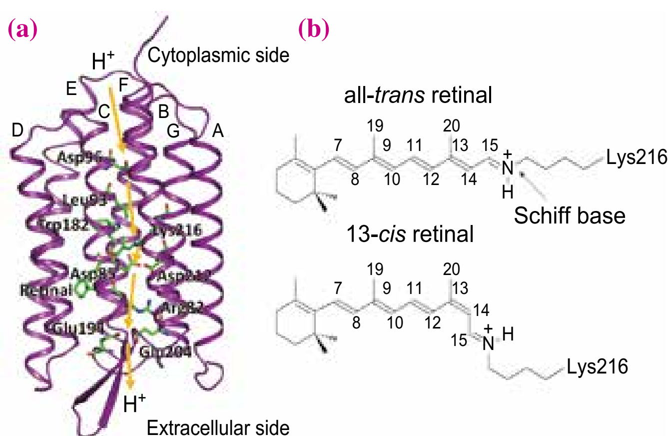

Fig. 1. Structure and function of bacteriorhodopsin (bR). **(a)** Proton-exchange steps (arrows) achieving proton pumping by bR. **(b)** Schematic illustrating retinal covalently bound to Lys216 through a protonated Schiff base in the retinal.

Considerable effort has been made to understand how structural changes in bR transport a proton uphill against a transmembrane potential. Many research groups have performed cryo-trapping experiments on bR using synchrotron radiation sources, providing information about structural changes during the photocycle. Despite these successes, there were certainly weaknesses in these experiments. Intermediate trapping studies were performed at a low temperature and thus were not truly time-dependent. Furthermore, conventional crystallography is subject to radiation damage and early results have been criticized for this reason.

We circumvent these concerns by recording a three-dimensional movie of structural changes in bR at room temperature with 2.1 Å resolution using time-resolved (TR) serial femtosecond crystallography (SFX) at SACLA **BL3**. The recent advent of intense femtosecond X-ray pulses from an X-ray free electron laser (XFEL) has enabled to acquire diffraction patterns from protein microcrystals before the onset of radiation damage. In SFX, microcrystals are continuously delivered with a solvent or a carrier media by an injector, allowing the observation of a damage-free structure at physiological temperature [1]. We used an optical-fiber-based setup for nanosecond pump-probe TR-SFX to visualize conformational changes in bR [2]. In TR-SFX, a continuous stream of microcrystals is injected across a focused XFEL beam, and the delay between sample photoactivation and the arrival of an XFEL pulse is controlled electronically.

TR-SFX data were collected from light-adapted bR microcrystals after photoactivation by a nanosecond laser pulse for $\Delta t = 16$ ns, 40 ns, 110 ns, 290 ns, 760 ns, 2 μs, 5.25 μs, 13.8 μs, 36.2 μs, 95.2 μs, 250 μs, 657 μs, and 1.725 ms. Our data revealed that the retinal is initially tilted in response to photoisomerization at $\Delta t = 16$ ns, and a water molecule (W402) is rapidly disordered by retinal isomerization (Fig. 2(b)). Low-temperature trapping studies have suggested that W402 disorders upon retinal photoisomerization [3], but this conclusion was challenged in light of similar

observations due to X-ray-induced radiation damage. Because no effects of radiation damage are visible when using <10 fs XFEL pulses at an X-ray dose of 12 MGy [4], these TR-SFX data bring closure to this debate.

Retinal isomerization reorients the Schiff base proton into a hydrophobic cavity while breaking its hydrogen bond to W402, both of which lower the proton affinity of the Schiff base. An initially twisted retinal becomes planar within 290 ns, causing Trp182 and Leu93 to be displaced toward the cytoplasm. These displacements allow a water molecule (W452) to order between Leu93, Thr89, and the Schiff base, which is observed for 40 ns $\leq \Delta t \leq$ 13.8 μs but W452 disappears after a proton is transferred to Asp85 (Fig. 3(a)). Hydrogen-bond interactions from the protonated Schiff base to W452 or Thr89 create a pathway for proton transfer to Asp85 and explain how the Schiff base comes in contact with Asp85 despite having been turned toward the cytoplasmic side by photoisomerization.

On the extracellular side of the retinal, the disordering of W402 triggers the disordering of W400 and W401 and the ordering of a new water molecule, Wat451, between Asp85 and Asp212 from $\Delta t \geq$ 13.8 μs (Fig. 3(b)). These water rearrangements allow that the extracellular portions of helix C bend toward helix G approximately 10 μs after photoactivation, which raises the p*Ka* of Asp85 to the point where it may spontaneously accept a proton from the Schiff base. Consequently, the time required for bR to evolve to a conformation with helix C bent toward helix G is the rate-limiting step that controls the primary proton transfer and explains why it takes microseconds for the Schiff base to be deprotonated.

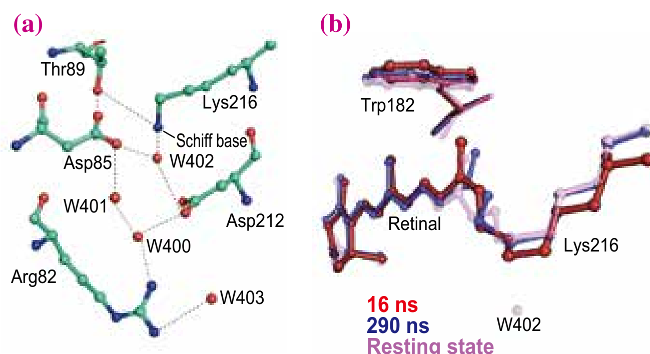

Fig. 2. Early structural changes in the bR photocycle. **(a)** Key amino acid residues and water molecules for the proton transfer in the resting conformation. **(b)** Crystallography models derived from partial-occupancy refinement for Δt = 16 ns (blue) and Δt = 290 ns (red) superimposed on the resting bR structure (purple, partially transparent).

Furthermore, we found that once a proton is transferred, the hydrogen-bond interaction between Asp85 and Thr89 is lost, which breaks the connectivity to the extracellular side of the protein, enabling unidirectional proton transport (Fig. 3(b)). The resulting cascade of structural changes throughout the protein provides an unprecedented insight into how structural changes in bR conspire to achieve unidirectional proton transport.

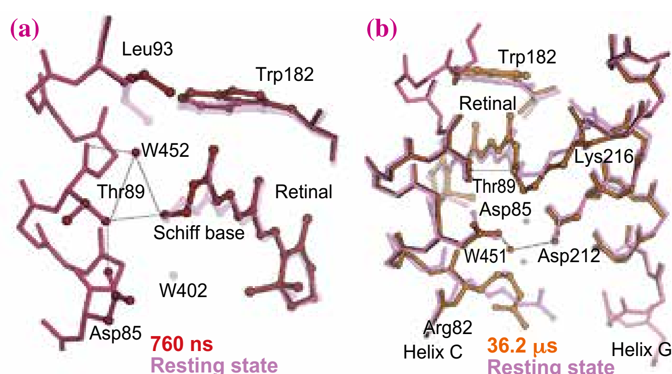

Fig. 3. Structural changes for Δt = 760 ns and Δt = 36.2 μs in the bR active site. **(a)** Crystallographic model for the time point Δt = 760 ns (red) superimposed upon the resting-state model (purple, partially transparent). **(b)** Crystallographic structural models derived from partial-occupancy refinement superimposed on the resting bR structure (purple, partially transparent) for Δt = 36.2 μs (orange).

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