High resolution structure analysis of bacteriorhodopsin K intermediate

Bacteriorhodopsin (bR) of *Halobacterium salinarum* is a proton pumping membrane protein driven by light energy (Fig. 1(a)) [1]. bR contains a retinal chromophore with a long conjugated double bond. The retinal connects to Lys216 of bR via a Schiff base linkage and is converted to the 13-*cis* form via photoisomerization while it is in the all-*trans* form in the resting state (Fig. 1(b)). The K intermediate is the first intermediate generated immediately after retinal isomerization. The structural changes associated with K formation are limited to retinal and the surrounding residues. Therefore, the K structure can provide insights into the proton pump mechanism in terms of energy storage and propagation in photoactive proteins. However, despite their importance, many inconsistencies have been observed among the previously reported K structures, especially in the conformation of retinal and interactions with surrounding residues (Fig. 1(c)). Therefore, we performed high resolution X-ray crystallographic analysis of the K intermediate [2].

Large bR crystals (approximately 300×300×30 μm^3) were prepared using the lipidic cubic phase (LCP) method, and the LCP matrix around the crystals was removed using squalane [3]. Diffraction experiments were performed at SPring-8 **BL41XU**. The K intermediate accumulated in the crystals upon irradiation with green laser light (λ = 532 nm) at 100 K. The diffraction dataset containing the K intermediate was collected at 15 K using He gas cooling. An additional diffraction dataset for the ground state was subsequently collected from the identical crystals at 15 K, after regenerating the ground state by irradiating with red laser light (λ = 678 nm) at 100 K. The X-ray absorption dose for the each data set was suppressed to be 0.05 MGy, which is one third of the limit at 15 K [3]. Both datasets indicated a resolution of approximately 1.3 Å.

In the F_o(K+bR) – F_o(bR) difference Fourier map, strong densities were observed only around retinal (Fig. 2). The 13-*cis* retinal in the K intermediate adopts an S-shaped conformation. The largest discrepancies from previously reported K structures occurred at the positions of the C13, C14, and C20 atoms of retinal. Regarding the interaction between retinal and the surrounding residues, the side chain of Lys216, which is covalently bound to retinal, interacts with Asp85 and Thr89 in the K intermediate. Furthermore, the Nζ-H bond of the protonated Schiff base linkage interacts with Asp212 and a water molecule, W402. The hydrogen bonding between Nζ and W402 in the K intermediate suggests that W402 moves to the cytoplasmic side of the Schiff base linkage via the hydrogen bond at the transition to the next L intermediate in the photocycle of bR.

The non-covalent interaction (NCI) plot shows the reduced density gradient isosurface [4]. The plot for K was calculated from a hydrogen atom-added structure obtained by quantum mechanics/molecular mechanics (QM/MM) calculations and used to examine the interactions around the Schiff base linkage (Fig. 3). Bluish green surfaces indicating the presence of relatively strong interactions are observed between the amide-proton on Nζ of Lys216 and Oδ1 of Asp212 as well as between Nζ of Lys216 and W402, as suggested directly from the crystal structure. In addition, green surfaces indicating the presence of

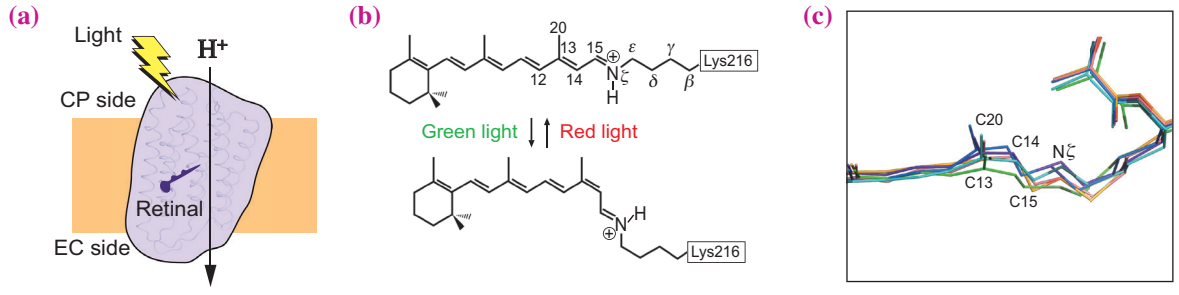

Fig. 1. Function of bR. (**a**) bR transports protons (H^+) from the cytoplasmic (CP) side to the extracellular (EC) side of the plasma membrane of *H. salinarum* using light energy. (**b**) All-*trans* retinal of bR in the resting state is converted to the 13-*cis* form by green light, while a back reaction is caused by red light at ~100 K. (**c**) Superimposition of retinal in various previously reported K structures. 1QK0, 1M0K, 1IXF, 6G7K, 6GA6 and 7Z0C are represented in green, yellow, cyan, dark blue, orange, and purple, respectively.

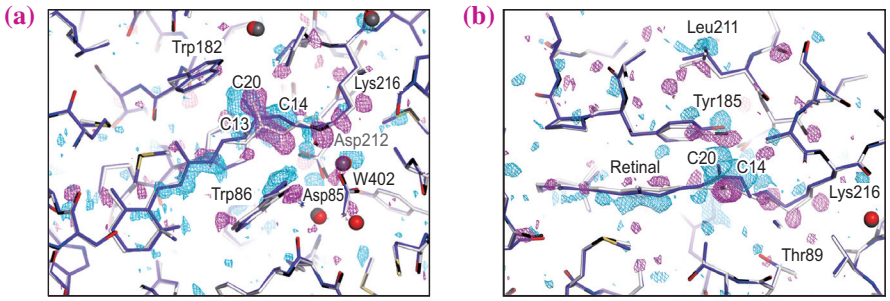

Fig. 2. Structural differences between the K intermediate and ground state. (**a**) The F(K+bR) – F(bR) map around retinal is represented by cyan (+3σ) and magenta (–3σ) meshes. The K structure is represented by violet sticks, while the ground state structure is superimposed as gray sticks. (**b**) View from the upper part of panel (a).

weak attractive interactions are observed between the methylene hydrogen atoms of Cε of Lys216 and the oxygen and hydrogen atoms of Thr89, as well as between Cε of Lys216 and Oδ1 of Asp85. These interactions are plausible stabilizing factors for the distorted conformation of retinal in the K intermediate.

The K structure in this study exhibited some differences from the recent time-resolved serial femtosecond crystallography (TR-SFX) K structures, such as the retinal conformation and water positions. In TR-SFX experiments, the contribution of multiphoton processes owing to the intense excitation laser pulses may pose a serious problem [5]. However, in this study, the K intermediate accumulated under light intensity as weak as sunlight by employing the cryo-trapping method. Therefore, multiphoton absorption could not occur. To further elucidate the proton pump mechanism of bR, the validity of the TR-SFX analyses with high-intensity pump lasers should be examined using integrated results, including those of this study.

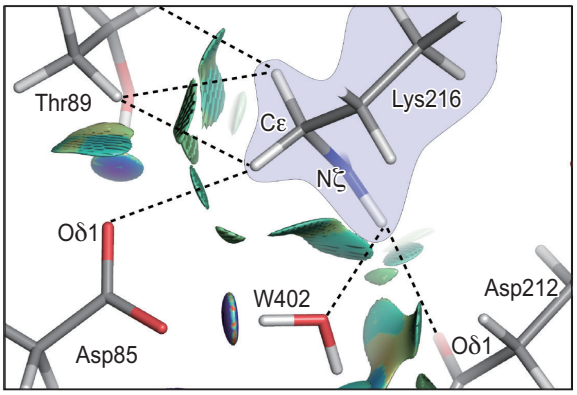

Fig. 3. NCI plot around the Schiff base linkage. The isosurface is colored according to a blue-green-red scheme, where blue, green, and red indicate attraction, very weak attraction, and repulsion, respectively.

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