Structure of the L Intermediate of Bacteriorhodopsin

Bacteriorhodopsin (bR), a membrane protein found in the cell membrane of *Halobacterium salinarum*, functions as a light-driven proton transport. Its polypeptide chain is folded into 7 transmembrane helices and retinal is bound to the ε-amino group of Lys216 *via* a protonated Schiff base linkage (Fig. 1). The photoisomerization of the all-*trans* retinal into the 13-*cis*, 15-*anti* configuration initiates a reaction cycle involving several intermediates ($bR_{570} \rightarrow K_{590} \rightarrow L_{550} \rightarrow M_{410} \rightarrow N_{560} \rightarrow O_{630} \rightarrow bR_{570}$). For the elucidation of the proton pumping mechanism, it is important to obtain structural information of the reaction intermediates. Using a 3D crystal belonging to the space group P622, we have previously determined the structures of the ground state (bR_{570}) and the K and the M intermediates at 2.3 - 2.6 Å resolutions [1,2]. Our structural data show that, in the K intermediate, the retinal chromophore has a largely twisted 13-cis, 15-anti configuration. The distortion in the retinal polyene chain is suggested to induce a vertical movement of helix G upon the formation of the M intermediate, which is accompanied by a large rearrangement in the hydrogen-bonding network in the proton release channel.

An increasing number of experimental data has shown that internal water molecules play an important role in regulating the pK_a values of key residues in the pathway of proton transport. The current structural model of the unphotolysed state (bR_{570}) shows that water molecules existing in the active site participate in stabilizing the protonated Schiff base with a very high pK_a (~ 13) and its counter ion (unprotonated Asp85) with a very low pK_a (~ 2.5). Recent FTIR studies of the L intermediate have suggested that, before the primary proton transfer, rearrangements of internal water molecules take place in such a manner that they cause a significant reduction in the pK_a of the Schiff base and a concomitant increase in the pK_a of Asp85. However, the structure of a key intermediate, i.e., the L intermediate, has not yet been determined convincingly, making it difficult to quantitatively analyze the detailed movements of internal water molecules.

For the structural investigation of the L intermediate [3], we carried out a quantitative analysis for X-radiation damage and searched for optimum conditions for X-ray measurements to minimize undesired effects of X-ray-induced structural changes. After a careful structural analysis, we constructed a structural model of the L intermediate at 2.4 Å resolution. Briefly, the P622 crystal was illuminated with green light at 160 K and subsequently with red light at 100K. This yielded a ~1:4 mixture of the L intermediate and the ground state. Diffraction data from such crystals were collected using a low flux of X-rays (~2×10^{15} photons/mm^2 per crystal), and their combined data were compared with those from unphotolyzed crystals. Figure 2 shows a difference electron-density map between the L intermediate and the ground state. These structural data, together with our previous data [1], indicate that the retinal chromophore, which is largely twisted in the K intermediate, takes a more planar 13-cis, 15-anti configuration in the L intermediate. This configurational change, which is accompanied by the reorientation of the Schiff base N-H bond towards the intracellular side, is coupled with a large rotation of the side chain of an amino acid residue (Leu93) making contact with the C13 methyl group of retinal. Following these motions, a water molecule, at first hydrogen-bonded to the Schiff base

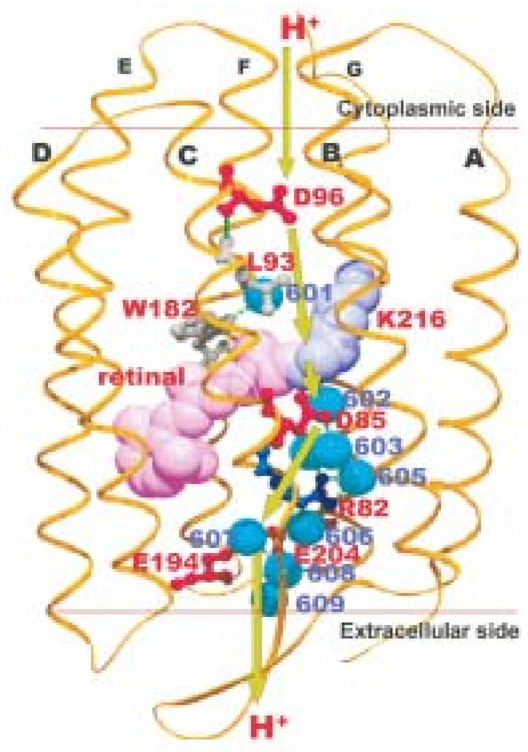

Fig. 1. Crystal structure of the ground-state of bacteriorhodopsin. Internal water molecules (cyan), the retinal-Lys216 chain (pink and light blue) and several key residues (red, blue and gray) are drawn. The green arrows indicate the pathway of proton translocation.

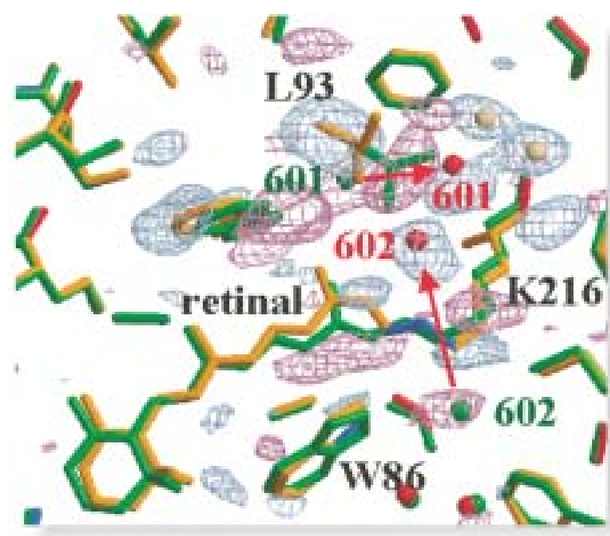

Fig. 2. Difference map between L and bR_{570}, contoured at 3.5σ (cyan, positive density; purple, negative density) and overlaid on the structural models of the ground state (green) and the L intermediate (carbon, oxygen and nitrogen atoms are drawn in orange, red and blue, respectively). Water molecules in L are represented by red (high occupancy) or gray spheres (low occupancy).

and Asp85, is dragged to a space that is originally occupied by Leu93.

In Fig. 3, the movements of water molecules observed in the bR_{570}-to-K and K-to-L transitions are summarized. A small displacement of Wat602 (i.e., towards the C^{δ} of Lys216) is already initiated in the primary photoreaction, which is accompanied by the isomerization of the retinal into a largely twisted 13-*cis*, 15-*anti* configuration. A large jump of Wat602 from the extracellular side of the Schiff base to the opposite side is assisted by the ~ 90° rotation of the N-H bond of the retinal Schiff base.

Now, an interesting question rises as to why the vertical movement of a key water molecule takes place prior to the primary proton transfer (from the Schiff base to Asp85), which takes place in the L-to-M transition. Since the retinal chromophore is accommodated tightly in a hydrophobic pocket, there is no channel for the passive movement of water across a horizontal plane passing through the chromophore. This plane can act as a structural barrier against the movement of water. Probably the backward translocation of Wat602 would not occur unless the cis-to-trans isomerization of the retinal is initiated by the excitation of the L intermediate.

Indeed, diffraction data from a crystal containing the M intermediate showed that this water molecule moves further towards the intracellular side in the L-to-M transition [3]. It is very likely that the detachment of this water molecule from the protonated Schiff base causes a significant decrease in the pK_a of the Schiff base, thereby facilitating the proton transfer to Asp85. On the basis of these observations, we argue that the vertical movement of a water molecule in the K-to-L transition is a key event determining the directionality of proton translocation in the protein.

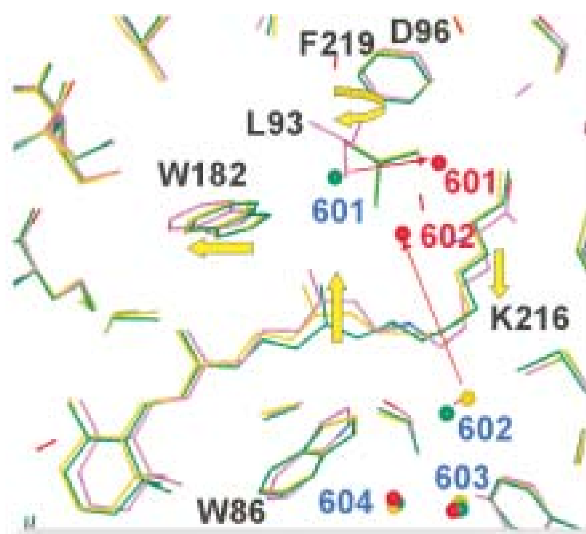

Fig. 3. Movements of key water molecules (indicated by thin arrows) and conformational changes in Leu93, Trp182 and the retinal-Lys216 chain (indicated by thick arrows) during the $bR_{570} \rightarrow K$ and $K \rightarrow L$ transitions. Atoms in bR_{570}, K and L are drawn in different colors.

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