Structural Changes during the Formation of Early Intermediates in the Bacteriorhodopsin Photocycle

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April 25, 2002

Running title: Early Intermediates of bR’s Photocycle

Key words: K, KL, hydrogen-bond network, excitation energy, molecular dynamics simulation, quantum mechanical/molecular mechanical calculation

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Abstract

Early intermediates of bacteriorhodopsin’s photocycle were modeled by means of ab initio quantum mechanical/molecular mechanical (QM/MM) and molecular dynamics (MD) simulations. The photoisomerization of the retinal chromophore and the formation of photoproducts corresponding to the early intermediates were simulated by MD simulations. By means of the QM/MM method, the resulting structures were refined and the respective excitation energies were calculated. Two sequential intermediates were found with absorption maxima that exhibit red-shifts from the resting BR state. The intermediates were therefore assigned to the K and KL states. In K, the conformation of the retinal chromophore is strongly deformed, and the N–H bond of the Schiff base points almost perpendicular to the membrane normal toward Asp212. The strongly deformed conformation of the chromophore and weakened interaction of the Schiff base with the surrounding polar groups are the means by which the absorbed energy is stored. During the K-to-KL transition, the chromophore undergoes further conformational changes that result in the formation of a hydrogen bond between the N–H group of the Schiff base and Thr89, as well as other rearrangements of the hydrogen-bond network in the vicinity of the Schiff base, which are suggested to play a key role in the proton transfer process in the later phase of the photocycle.
1 Introduction

Bacteriorhodopsin (bR), a transmembrane protein residing in the purple membrane of *Halobacterium salinarum*, functions as a light-driven proton pump to produce a proton gradient across the membrane (Oesterheldt and Stoeckenius, 1971). The protein consists of seven transmembrane α-helices (Henderson and Unwin, 1975) surrounding an all-trans retinal chromophore covalently bound to Lys216 through a protonated Schiff base linkage. Figure 1 depicts the chemical structure of the protonated all-trans retinal Schiff base and its link to the protein.

Retinal contains six conjugated double bonds including the Schiff base group.

Upon absorption of light in the visible spectral region, the retinal chromophore undergoes in bR an isomerization around the C$_{13}$=C$_{14}$ double bond, resulting in a 13-cis configuration. The photoisomerization initiates a photocycle during which bR completes an active proton transport event across the membrane from cytoplasm to the extracellular side. The photocycle comprises a series of intermediates, labeled K, L, M, N, and O, characterized by their absorption maxima, and is completed through return to the initial (BR) state (Lozier et al, 1975). The lifetimes of the intermediates range from subpicoseconds to miliseconds. The photocycle involves protein structural changes, which are coupled to proton transfer between such key residues as the retinal Schiff base, Asp85, Asp96, and Glu204 (Braiman et al., 1989; Gerwert et al., 1989; Brown et al., 1995). A sequence of proton transfer events between these residues completes the overall uni-directional proton pump through the channel.

The three dimensional atomic structure of the resting BR state has been determined by
cryoelectronic microscopy (Grigorieff et al., 1996; Mitsuoka et al, 1999) and x-ray crystallography (Pebay-Peyroula et al., 1997; Luecke et al., 1998; 1999; Belrhali et al., 1999). These structures reveal the position of key residues facing the interior of the protein and forming the proton channel. The protonated Schiff base group lies in the middle of the channel dividing it into the cytoplasmic and extracellular half-channels. High resolution x-ray structures (Luecke et al, 1999a; Belrhali et al, 1999) have also shown a hydrogen-bond network (HBN) in the extracellular half of the channel including several internal water molecules. The existence of water molecules was first suggested by nuclear magnetic resonance (NMR) (de Groot et al., 1989) and Fourier transform infrared (FTIR) spectroscopic studies (Maeda et al., 1994; Fischer et al., 1994; Kandori et al, 1995; Chon et al., 1996). In Fig. 2a, the structure of the retinal binding site of the x-ray structure of BR (Luecke et al., 1999a. PDB code: 1C3W) is shown. Figure 3 illustrates the topology of the HBN in the retinal binding pocket, including the negatively charged Asp85 and Asp212, the Schiff base, and three internal water molecules (Wat401, Wat402, and Wat406).

After photoisomerization, a bathochromic (red-shifted) intermediate, called K, forms in 3 ps. The K state that is trapped at liquid nitrogen temperature 77 K is called KLT. In K, the energy of the absorbed photon is stored in a high energy structure, the relaxation of which induces a well-controlled series of proton translocations. The stored energy has been estimated to be 16 kcal/mol by photocalorimetric experiments (Birge and Cooper, 1983; Birge et al., 1989). The structural changes during the K formation have been extensively investigated by various
methods. Low temperature resonance Raman (LT-RR) and low temperature FTIR (LT-FTIR) spectroscopic studies have shown significant chromophore conformational deformations in \( K_{LT} \) (Pande et al., 1981; Braiman and Mathies, 1982; Bagley et al., 1982; Rothschild and Marrero, 1982). LT-FTIR studies have also revealed alterations of the HBN in the vicinity of the Schiff base during the K formation (Kandori et al., 1998a; 1998b; 1999; 2000; 2001a; Kandori and Shichida, 2000).

Recently, an x-ray crystallographic structural model of the \( K_{LT} \) intermediate has been reported (Edman et al., 1999). Figure 2b shows the retinal binding site of the x-ray structure (PDB code: 1QKO). The K–BR difference electron density map reported in that study (Edman et al., 1999) clearly shows a strong negative density at the position of Wat402 in BR, indicating the dislocation of this water molecule during the formation of \( K_{LT} \). The density map also reveals a remarkable conformational change of Asp85, which leads to the complete dissociation of its hydrogen-bond with Thr89 and its closer distance to the Schiff base. The authors have argued that these structural changes play a key role in the primary proton transfer between the Schiff base and Asp85 in a later (L-to-M) step. However, due to the weak electron density for \( K_{LT} \), the position of the dislocated W402 and the conformation of the chromophore in \( K_{LT} \) have not been determined clearly. Moreover, the complete dissociation of the hydrogen-bond between Asp85 and Thr89 disagrees with recent LT-FTIR results that indicate a stronger hydrogen-bond between these groups upon the formation of \( K_{LT} \) (Kandori et al., 1998b; 1999; 2001a).
Two distinct K-like bathochromic intermediates have been resolved by visible (Shichida et al., 1983), FTIR (Rothschild et al., 1985; Sasaki et al., 1993; Weidlich and Siebert, 1993; Hage et al., 1996; Dioumaev and Braiman, 1997), and resonance Raman (Althaus et al., 1995; Doig et al., 1991) spectroscopic measurements. These intermediates appear sequentially in time, and the early and late ones have been conventionally called K and KL, respectively. The formation of KL at room temperature has been observed in $\sim 10$ ns by the visible absorption measurements (Shichida et al., 1983), and in 50–100 ns by the time resolved FTIR (TR-FTIR) experiments (Hage et al., 1996; Dioumaev and Braiman, 1997). Although the spectroscopic studies have suggested significant structural changes involved in the K-to-KL transition, the KL intermediate has not been well understood at the atomic level.

The structure and dynamics of bR have been investigated by many theoretical studies (Schulten et al., 1995; Logunov and Schulten, 1996; Xu et al., 1996; Humphrey et al., 1998; Ben-nun et al., 1998; Tajkhorshid et al., 2000; Baudry et al., 2001; Warshel et al., 1991; Warshel and Chu, 2001; Nina et al., 1995; Yamato and Kakitani, 1997). Recently, ab initio quantum mechanical/molecular mechanical (QM/MM) calculations have been applied to investigate the structure, the proton transfer reaction, and the electronic and vibrational absorptions of the BR state (Hayashi and Ohmine, 2000).

QM/MM methods have been widely used to study chemical reactions in solution phase and in proteins (Warshel and Levitt, 1976; Warshel et al., 1991; Warshel and Chu, 2001; Field et al., 1990; Gogonea et al., 2001; Maseras and Morokuma, 1995; Merill and Gordon, 1998; Gao
and Xia, 1992; Logunov and Schulten, 1996). In QM/MM methods, the part of the system that involves electronic changes is described quantum mechanically (QM), and the rest, which provides steric and electrostatic environmental effects on the QM part, is treated by a molecular mechanical (MM) force field.

Hayashi and Ohmine (2000) computed the proton transfer potential energy profile in bR by QM/MM calculations and showed that the primary proton transfer can be efficiently triggered by a certain rearrangement of the HBN, which can be induced by the chromophore photoisomerization (Hayashi and Ohmine, 2000). The authors have also described quantum mechanically the electronic structures of the chromophore and the HBN in the vicinity of the Schiff base, which is closely involved in the proton transfer process. The electronic nature of the HBN results in anomalous IR signals of the O–D and N–D stretching modes that have been confirmed by a recent IR measurement (Kandori et al., submitted). The excitation energy of the chromophore in the protein matrix was also calculated and analyzed in terms of the chromophore-protein interactions. The ab initio QM/MM approach has also been applied to identify the molecular mechanism of its prominent absorption spectral features of sensory rhodopsin II (Hayashi et al., 2001), for which x-ray crystallographic structures have been determined recently (Royant et al., 2001; Luecke et al., 2001).

In the present paper, we report molecular dynamics (MD) simulations and ab initio QM/MM calculations modeling the early intermediates of bR’s photocycle. The photoisomerization and the formation of the photoproducts, i.e., the early intermediates, were simulated by MD. As
mentioned above, a proper description of electronic effects of the chromophore and the HBN is crucial for the study of the chromophore–protein interactions and their dynamics. Thus we introduce a polarizable water model (Rick et al., 1994) for internal water molecules and reparameterized the force field functions by the QM/MM method. The structures of the photoproducts obtained by MD simulations are further refined by QM/MM full geometry optimizations, where the chromophore and the HBN are calculated fully quantum mechanically. At the optimized structures of the intermediates, electronic excitation energies of the chromophore were also calculated in order to verify the identified intermediates and to elucidate the molecular basis of their bathochromic shifts. The present study provides detailed atomic structural models of the K and KL intermediates and suggests key structural changes in the proton pump process. The study also addresses discrepancies between the x-ray structural model and the spectroscopic observations.

2 Methods

In this section, details of preparation of the structural model will be first presented. Then, we will describe protocols and potential energy functions used for MD simulation of the chromophore isomerization and the formation of intermediates. Finally, methods of the QM/MM geometry optimization, energy decomposition analysis, and excitation energy calculations will be described.
2.1 Structural model

A structural model of the ground state (BR) was constructed based on the high resolution crystal structures (Luecke et al., 1999a; Belrhali et al., 1999). The model includes nine water molecules: two in the cytoplasmic half of the channel, three in the Schiff base binding site (Wat401, Wat402, and Wat406), one between Arg82 and Thr205, and three in the vicinity of Glu194 and Glu204. Asp96, Asp115, and Glu204 were protonated, and the other acidic and basic residues were assumed to be charged (Sasaki et al., 1994; Brown et al., 1995).

2.2 MD simulation

MD simulations were used to model the chromophore photoisomerization and to obtain starting structural models of the early intermediates, which were refined by the QM/MM optimization described in Sec. 2.3. The AMBER force field (Cornell, 1995) was used for the standard protein residues, whereas force fields of the retinal chromophore and water molecules were reparameterized based on the QM/MM methods, as described below. Time integration of the equation of motion was performed by the leap-frog algorithm with a 1 fs time step. The bond distances including hydrogen atoms were kept fixed by SHAKE. In order to maintain the overall shape of the protein, harmonic constraints with a force constant of 2.0 kcal/mol/Å² were applied to Cα atoms further than 12 Å from the retinal chromophore. This approximation is validated by electron microscopic (Bullough and Henderson, 1999) and x-ray crystallographic (Edman et al., 1999) studies, showing that no major conformational changes of helices accompany
the formation of K. A 12 Å residue-based cutoff was used for non-bonding interactions. The Berendsen method (Berendsen et al., 1984) was employed to control the temperature.

The BR ground state was equilibrated for 200 ps at 300 K starting from the QM/MM optimized structure (Hayashi and Ohmine, 2000), and then an equilibrium simulation of BR for 300 ps was performed to sample initial coordinates and velocities for the simulations of photoisomerization. The chromophore photoisomerization was simulated from the initial configurations using the potential energy function of the excited state, which steers the 13-trans excited state to the 13-cis ground state photoproduct (see below). During the isomerization, the temperature control was turned off. To determine energy minimized structures of the photoproducts from trajectories of the photoisomerization, the system was first cooled gradually to 50 K in 40 ps, and then subjected to the QM/MM optimization.

For the BR ground state, we employed the force field parameters of dihedral angles of the main polyene chain of retinal (Set B in Tajkhorshid et al., 2000) determined previously based on density functional calculations (Tajkhorshid, et al., 1999). In addition, improper torsional functions with $V_n/2 = 0.3$ kcal/mol were applied to represent $sp^2$ planarities in the conjugated polyene.

For the isomerization, the potential energy function of the C$_{13}$=C$_{14}$ dihedral angle, $\phi_{13=14}$, was expressed by seven Fourier components listed in Table 1. Figure 4 shows the energy profile of the potential function. The internal conversion to the 13-cis ground state is described by downhill dynamics on a single potential curve. In this description, the electronic details of
the transition process from the excited state to the ground state during the isomerization are
neglected. We assumed that the reactive trajectories to the $13$-cis configurations are not drasti-
cally perturbed by the electronic transition process, because the internal conversion likely takes
place through conical intersections, which are expected to be highly efficient doorways to the
ground state. The potential energy curve displays a plateau region around the $13$-trans config-
uration, as suggested by quantum chemical calculations (Garavelli et al, 1997; 1998; Humphrey
et al., 1998; González-Luque et al., 2000). Unfortunately, the accurate excited state potential
profile of the chromophore with six double bonds in the protein matrix has not been calcu-
lated yet. We therefore repeated the photoisomerization with several trial potential functions
that differ with respect to the width of the plateau region. Those different potential functions
produced almost the same photoproducts, although the isomerization rate was sensitive to the
width. The other dihedral parameters of the chromophore remained the same as those in the
ground state, except for $\text{C}_{15}=\text{N}_c$ and $\text{C}_{14}-\text{C}_{15}$, for which we employed single well potentials
with the same curvatures at the trans configurations to inhibit their rotations.

Recently, the reaction potential energy surfaces of retinal analogues from the Franck-Condon
region to the product state in isolated form have been precisely investigated by means of
high-level quantum chemical calculations (Garavelli et al., 1997; 1998; Humphrey et al., 1998;
González-Luque et al., 2000; Molnar et al., 2000). These studies showed the involvement of
the stretching motions of the polyene skeleton in the isomerization process, especially in the
initial 100–200 fs (the H-to-I process) and during the electronic transition at the conical in-
intersections. The quantum dynamics of the electronic transitions in the protein matrix has also been examined by semi-classical techniques (Humphrey et al., 1998; Ben-nun et al., 1998; Warshel and Chu, 2001) suggesting mechanisms accounting for the high quantum yield (0.64) of the isomerization process in bR. The present classical treatment of the isomerization by a one-dimensional potential function is a rather crude approximation and unable to reproduce prominent characteristics of the photoisomerization dynamics observed in ultrafast spectroscopic studies. Nevertheless, it seems to be unlikely that lack of the details of the actual photodynamics leads to a qualitatively irrelevant structural model of the primary photoprodut, because the conformational change of the chromophore during the isomerization is apparently dominated by the rotation around the C$_{13}$=C$_{14}$ bond. Moreover, the quality of the structures of the photoproducts, which may be compromised by the applied simple potential functions of the chromophore, is improved through the QM/MM structure refinements that follow the MD simulations in the present modeling scheme.

It is well-known that the charge distribution of the retinal chromophore in bR largely changes both during the photoexcitation and isomerization. Therefore, we employed effective charge parameters for the chromophore in the ground and excited states, $q_{i}^{g}$ and $q_{i}^{e}$, respectively, derived from QM/MM complete active space self-consistent field (CASSCF) calculations performed at the ground state energy minimum geometry (Hayashi et al., 2001). These charges are listed in Table 2. The change of charge distribution during the isomerization was then
expressed by a function that analytically interpolates $q_i^{S_0}$ and $q_i^{S_1}$ smoothly at $\phi_{13=14} = 90^\circ$,

$$q_i = q_i^{S_0} \times \frac{1}{2} (1 - \tanh \Phi) + q_i^{S_1} \times \frac{1}{2} (1 + \tanh \Phi),$$  \hspace{1cm} (1)$$

$$\Phi = \frac{-|\phi_{13=14}| + 90}{a \times 90}. \hspace{1cm} (2)$$

$a$ was chosen to be 0.1, which gives an abrupt switch of the charges around $\phi_{13=14} = 90^\circ$ to mimic the so-called sudden polarization at the conical intersection. It is noted that the change of the charge distribution upon the excitation results in a difference of the chromophore-protein electrostatic interactions, $\Delta V_{ES}^{ch-pr}$ and, therefore, contributes to the $S_1 - S_0$ excitation energy (see Sec. 2.5). In the BR state, the $\Delta V_{ES}^{ch-pr}$ contribution for this effective charge parameter set was estimated to be 11.2 kcal/mol by our preliminary MD simulations. Therefore, the vertical energy difference of the internal potential functions at $\phi_{13=14} = 180^\circ$ was set to be 39.1 kcal/mol ($= 50.3$ kcal/mol (568 nm) – 11.2 kcal/mol) as shown in Fig. 4.

Rearrangement of the HBN in the proton channel plays an important role for the pump process. Therefore, a proper description of the HBN is also crucial in our MD simulation. As shown by a previous QM/MM study (Hayashi and Ohmine, 2000), there are strong electronic interactions, such as polarization and charge transfer, between the Schiff base, Asp85, and Wat402. The Kitaura-Morokuma energy decomposition analysis (Kitaura and Morokuma, 1976) showed that Wat402 has a polarization energy of $-14.1$ kcal/mol and a charge transfer energy of $-18.2$ kcal/mol due to its interaction with the Schiff base, Asp85, and other surrounding moieties. These electronic interaction energies are considerably larger than those in water-water interactions; for example, the polarization and charge transfer energies in a water
dimer are about −1 to −2 kcal/mol (Umeyama and Morokuma, 1977). In our preliminary MD simulations, we found that the TIP3P-AMBER force field, in which non-bonding interactions are described by only classical pair-wise potential functions, cannot even reproduce the conformation of Wat402 observed in the x-ray ground state structure; after the MD equilibration, the Schiff base N–H bond directly attaches to O$_{\delta^-}$ of Asp85, and Wat402 no longer bridges the two. We also observed in the QM/MM optimized ground state structure a strong interaction between N–H of the Schiff base and a lone pair orbital of Wat402 (Hayashi and Ohmine, 2000); the TIP3P water model cannot represent this hydrogen-bond correctly because of the poor description of the lone pairs and electronic interactions.

Figure 5 compares the interaction energies of Wat402 with its surroundings in bR, determined in the frame work of the TIP3P-AMBER force field, or by means of the QM/MM calculations. Details of the QM/MM calculations are described in Sec. 2.3. The energies were estimated for the energy minimum conformation, and for 26 other conformations generated by ±15° rotations of the W402 molecule around the Cartesian axes. As clearly seen, the TIP3P-AMBER energies strongly deviate from the QM/MM ones, indicating the poor orientational description of the TIP3P model due to lack of lone pair electrons and electronic interactions.

The potential functions describing the internal water molecules were therefore improved as follows. First, the fluctuating charge model (fq-SPC) developed by Rick et al. (1994) was used for all water molecules. This model can take into account the multi-body effect due to the electronic polarization induced by the polar environment. Furthermore, we added potential
energy terms representing explicitly the angular dependence of hydrogen-bonds and short-range attractions between Wat402 and the counter-ion aspartate groups. Parameters appearing in the additional potential energy terms were fitted so as to reproduce the QM/MM energies. The reparameterized force field is denoted as Set 1. The functional forms and parameters are summarized in an Appendix. Figure 5 also compares the interaction energies of Wat402 by using the Set 1 force field with the QM/MM values. One can see that the energies of the present model are in good agreement with those of the QM/MM results. Using this model for water, the hydrogen-bond network of the ground state remained stable during a 500 ps MD trajectory. In the simulations of photoisomerization, these additional functions were excluded for the excited state, and smoothly switched on during the isomerization with the same switching function used for effective charges [see Eqs. (1) and (2)]. In the simulations for the modeling of KL, we employed the fq-SPC model for Wat402, but removed the additional functions (see Sec. 3.3). This force field is denoted as Set 2.

### 2.3 QM/MM geometry optimization

The early intermediate structures obtained by the MD simulations were refined through QM/MM geometry optimizations. We employed a QM/MM method, described elsewhere (Hayashi and Ohmine, 2000), that allows one to determine efficiently the optimized geometry of the whole system. One-electron operators based on a restrained electrostatic potential (RESP) method (Bayly et al., 1993) were used to describe the electrostatic interaction between the QM and
MM regions, thus, avoiding the time consuming computation of electron integrals of gradient components due to the QM–MM interaction during the optimization of the MM region, which usually needs a large number of searching steps. The QM/MM method is implemented in the HONDO (Dupuis et al., 1992) program package. The AMBER force field (Cornell et al., 1995) and the Hartree-Fock (HF) level of theory were used for the description of the MM and QM regions, respectively. For the geometry optimization, the side chains of retinal-Lys216, Asp85, and Asp212, as well as three water molecules (Wat401, Wat402, and Wat406) were included in the QM part. Dunning’s split-valence (DZV) basis set (Dunning and Hay, 1977) was used, together with diffuse functions on the Oδ atoms of the aspartate side chains. The boundaries dividing the QM and MM regions were set at the Cβ–Cγ bond of Lys216 and the Cα–Cβ bonds of Asp85 and Asp212, and the open valences in the QM region were filled by dummy hydrogen atoms following the link atom approach (Field et al., 1990). The interactions between the dummy hydrogen atoms and the MM region were excluded by the RESP treatment (Hayashi and Ohmine, 2000). A 12 Å residue-based cutoff was used for non-bonding interactions.

2.4 QM/MM energy decomposition analysis

The QM/MM energies of the BR and intermediate states were analyzed by decomposing them into several contributions. The total energy of the QM/MM system is expressed as

$$E = E_{Q\text{M}/\text{MM}} + E_{\text{MM}},$$

(3)
where $E_{QM/MM}$ includes energies of the QM region itself and of the QM-MM interaction, and $E_{MM}$ consists of only interactions within the MM region. Unfortunately, it is rather difficult to consistently determine $E_{MM}$ of different states in the present scheme because of large fluctuations of polar residues around the surface regions. In this study, therefore, we examined only the $E_{QM/MM}$ term.

$E_{QM/MM}$ is decomposed into several terms:

$$E_{QM/MM} = E^0 + V^{ES}_{QM-MM} + V^{LJ}_{QM-MM} + V^{BAD}_{QM-MM} + E^{ERO}_{QM(MM)}.$$  (4)

$E^0$ is the QM energy in isolated condition computed at the geometry inside the protein matrix. $V^{ES}_{QM-MM}$ and $V^{LJ}_{QM-MM}$ are electrostatic and Lennard-Jones (LJ) interactions between the QM and MM regions, respectively. $V^{BAD}_{QM-MM}$ expresses the bonding contributions (bond, angle and dihedral (BAD)) at the QM/MM boundary. $E^{ERO}_{QM(MM)}$ is the electronic reorganization (ERO) energy. The external electrostatic field produced by the MM protein matrix polarizes the QM electronic wave function and naturally contributes to $\Delta V^{ES}_{QM-MM}$. The induced polarization, however, involves deformation of the electronic wave function, known as ERO, and therefore gives rise to a destabilization effect that to some extent compensates the electrostatic stabilizations (Gao and Xia, 1992; Gao, 1997).

We carried out this analysis for the same QM/MM system employed in the geometry optimizations. The QM region includes side chains of retinal-Lys216, Asp85, and Asp212, and three water molecules (Sec. 2.3). This QM/MM system is denoted as System A. We also applied the analysis to a QM/MM system in which only the side chain of retinal-Lys216 is included in the
QM region, denoted as System B. In both systems, the QM regions were described by the HF method with the DZV basis set.

2.5 QM/MM excitation energy calculations

In the description of the excited state, we employed the QM/MM method using the CASSCF level of theory. The excitation energy \( E_{ex} \) was carried out for System B (Sec. 2.4). Twelve electrons in eleven valence \( \pi \) orbitals of the chromophore were chosen for the CAS space. To improve the flexibility of basis functions, polarization functions were added to the C and N atoms of the conjugated backbone of the retinal Schiff base. The excitation energies were computed by the state-averaged CASSCF calculations for the lowest three \( \pi-\pi^* \) states (\( S_0 \), \( S_1 \), and \( S_2 \)), followed by the state specific CASSCF calculations for the \( S_0 \) and \( S_1 \) states.

In order to analyze the molecular mechanism of the characteristic bathochromic shifts of the intermediates, \( E_{ex} \) was also decomposed in the same way as described in 2.4. Since \( V_{QM-MM}^{LJ} \) and \( V_{QM-MM}^{BAD} \) do not change upon the excitations, \( E_{ex} \) is expressed by only three contributions (Hayashi et al., 2001),

\[
E_{ex} = E_{ex}^0 + \Delta V_{ch-pr}^{ES} + \Delta E_{ERO}^{ch(pr)}. \tag{5}
\]

Here \( E_{ex}^0 \) is the excitation energy in isolated condition; \( \Delta V_{ch-pr}^{ES} \) is the difference between the chromophore(ch)-protein(pr) electrostatic interaction energies in the \( S_0 \) and \( S_1 \) states; \( \Delta E_{ERO}^{ch(pr)} \) is the difference between the ERO energies in those states. Note that upon excitation the positive charge of the chromophore, which is mainly localized in the Schiff base region in the
ground state, migrates toward the β-ionone ring side of the polyene chain. Hence, the negatively charged counter-ion groups in the vicinity of the Schiff base stabilize the ground state more strongly than the excited state, and, consequently, $\Delta V_{\text{ch-pr}}^{ES}$ is a large positive contribution (Hayashi and Ohmine, 2000; Hayashi et al., 2001).

3 Results

MD simulations were performed to examine the photoisomerization and structural changes in the early intermediates, K and KL. Conformational changes of the retinal chromophore and rearrangements of the HBN in the retinal binding pocket revealed by the simulations and QM/MM optimizations are described below. The energetics of the K and KL formations are analyzed. The molecular mechanism of the absorption spectral shifts of the intermediates are also discussed.

3.1 Photoisomerization

Using different initial configurations (coordinates and velocities) selected from the ground state equilibrium trajectory at time intervals of 5 ps, forty isomerization MD trajectories, 20 ps long each, were calculated. After the photoexcitation, the trajectories stayed around the Franck-Condon region transiently before proceeding to the 13-cis configuration. The average lifetime of the excited state was 116 fs. Since in the present model, the vibrational relaxation of the
in-plane skeleton modes of the polyene part corresponding to the process from the Franck-Condon region (H) to a fluorescent state (I) is not described, the lifetime of the excited state is expected to be shorter than the experimental one (the formation time of the vibrationally hot photoproduct, J, is $\sim 500$ fs). The lifetime is rather sensitive to the potential profile describing torsion around the $C_{13}=C_{14}$ bond and precise molecular models are therefore needed to reproduce the experimentally observed lifetime.

The simulations provided an insight into the directionality of the isomerization, i.e., clockwise or counter clockwise around the $C_{13}=C_{14}$ bond. The two possible directions of the isomerization result in the rotation of the N–H bond of the Schiff base toward Asp85 or toward Asp212 (Fig. 4), respectively. In our simulations, all isomerizations happened toward Asp212. The unidirectional isomerizations toward Asp212 have been also reported in a recent computational study by Warshel and Chu (2001).

Since a symmetric function has been used for the description of the $C_{13}=C_{14}$ dihedral potential (Fig. 4), the uni-directionality of the isomerization is purely due to forces of the environment. Our previous MD and QM/MM studies showed that the conformation of the $C_{13} \sim N_{\zeta}$ part of the chromophore in the BR ground state is twisted and the N–H bond of the Schiff base is tilted toward Asp212 (Tajkhorshid et al., 2000; Hayashi and Ohmine, 2000). Particularly, the $C_{12}–C_{13}=C_{14}–C_{15}$ dihedral angle deviates considerably ($19^\circ$ in the QM/MM optimized geometry) from planarity. A twisted conformation of retinal toward Asp212 in BR, which was also observed in the present study, originates from the forces of the environment that favor the
isomerization in the direction of Asp212.

The unidirectional torsion arises from two kinds of interactions of retinal with the surroundings. One is the steric interactions with residues constituting the retinal binding pocket. Since the binding pocket exhibits a slightly bent shape, the conjugated chain of retinal adjusts itself to the binding pocket by twisting around the \( \text{C}_{13}=\text{C}_{14} \) and \( \text{C}_{15}=\text{N}_{\xi} \) double bonds (Tajkhorshid et al., 2000; Hayashi and Ohmine, 2000). Previous MD simulations (Tajkhorshid et al., 2000) have clearly shown that reduction of the bend of the binding pocket by replacing Trp86 by Ala results in the relaxation of torsion at those double bonds. The other interaction is due to the strong hydrogen-bond of the Schiff base N–H bond with Wat402. The resulting twisted structure of the chromophore could not be observed, when Wat402 was described by the TIP3P model, instead the present water model which includes three body effects and explicit interaction of lone pairs. Therefore, strong hydrogen-bonds between the Schiff base, Wat402, and Asp85 are essential for the twisted retinal conformation that also induces a unidirectional photoisomerization.

3.2 The K intermediate

In order to proceed to the first intermediate, eight isomerization trajectories were continued for 200 ps. All runs resulted in almost the same 13-cis photoproduct, in which the chromophore had a strongly deformed structure. Figure 6b displays the QM/MM energy minimized structure of this photoproduct, assigned to the K (or \( \text{K}_{LT} \)) intermediate. One can clearly see that the
chromophore is largely twisted in the C_{13} \sim N_{\zeta} region, and the N–H bond of the Schiff base is almost perpendicular to the membrane normal and pointing toward Asp212. In a fully planar 13-\textit{cis} conformation of the chromophore, the N–H bond is expected to point “up” (parallel to the membrane normal). However, in none of the eight simulations performed at room temperature, a completely relaxed planar 13-\textit{cis} conformation was observed. The N–H bond maintains its interaction with Wat402 and Asp212 in the K intermediate, thus preventing the chromophore from relaxing into a planar conformation.

Table 3 compares geometrical parameters of the chromophore in the QM/MM optimized structures of BR and K. In K, the strong torsions are mainly localized in the C_{13} \sim N_{\zeta} region. Especially, the C_{14}–C_{15}=N_{\zeta}–C_{\varepsilon} and C_{12}–C_{13}=C_{14}–C_{15} double bonds are largely twisted by 30° and 25°, respectively. Although dihedral angles of the other half of the polyene chain, C_{13} to C_{5}, do not significantly deviate from 180°, small rotations around C_{9}=C_{10} and C_{11}=C_{12} result in an overall twist in this half. Consequently, the C_{20} methyl group attached to C_{13} is tilted toward Asp212, and the angle of the C_{13}–C_{20} bond to the membrane normal in K differs by 30° from that in the BR ground state (for the numbering of retinal carbons, see Fig. 1).

Figure 7a schematically depicts the HBN in the K intermediate. The HBN does not undergo large conformational changes upon the formation of K, as can be discerned by comparison of Figs. 3 (BR) and 7 (K). Only minor changes in the hydrogen-bond distances were found, except for the hydrogen-bonds between H_{\zeta} of the Schiff base and Wat402. The isomerization of the chromophore moves the H_{\zeta} atom upward, resulting in a weakened hydrogen bond between the
Schiff base and Wat402. The distance between Wat402:H$_2$ and Asp85:O$_{\delta 1}$ also slightly increases due to the reduction of charge transfer and polarization effects from the Schiff base.

The strongly twisted chromophore in the present K intermediate presents a remarkable difference from the x-ray crystallographic model of Edman et al. (1999), in which the chromophore conformation is assumed planar. Furthermore, the K intermediate in the present study involves only minor changes of the HBN, as seen in Fig. 6, whereas in the x-ray model (Fig. 2b) significant changes of the HBN are observed as discussed in Sec. 1. In the present K model, Wat402 occupies almost the same position as in BR, and the hydrogen-bond between Thr89 and Asp85 is not broken.

The present K model is, however, in keeping with the results of LT-RR (Pande et al., 1981; Braiman and Mathies, 1982) and LT-FTIR (Bagley et al., 1982; Rothschild and Marrero, 1982) spectroscopic measurements, which indicate a strong enhancement of the HOOP vibrational peaks of the chromophore upon the formation of K. One of the prominent peaks at $\sim$ 960 cm$^{-1}$ has been assigned to the 15–HOOP mode (Braiman and Mathies, 1982). The intensities of the HOOP modes are small in the planar conformation and become large when the structure is distorted. Hence the torsional deformation around the Schiff base in our K model is consistent with the large HOOP peaks in the LT-RR and LT-FTIR spectra.

The IR spectral shape of the HOOP peak at 960 cm$^{-1}$ is altered by the mutation of Thr89 to Cys (Kandori et al., 1999). The present model of K can explain this mutation effect. The H$_{15}$ atom is close to Thr89:O$_{\gamma}$ (2.58 Å), and replacement of the O atom by the S atom, which is
larger, in the T89C mutant can affect the spectral shape of the 15–HOOP. Contrary to the x-ray model (Edman et al., 1999), FTIR measurements (Kandori et al., 1998b; 1999; 2001) indicate that the hydrogen-bond between Thr89 and Asp85 becomes stronger upon the formation of K. The distance between Thr89:H$_\gamma$ and Asp85:O$_\delta$ in the simulated K intermediate is 0.01 Å shorter than that in BR (Figs. 3 and 7a), implying a stronger hydrogen-bond, and thus supporting the FTIR result. The almost perpendicular orientation of the N–H bond of the Schiff base to the membrane normal is also in good agreement with a recent polarized FTIR measurement (Kandori et al., submitted).

3.3 The KL intermediate

As discussed above, the chromophore has a strongly twisted conformation in K because the Schiff base maintains its connection to Wat402. Nevertheless, the hydrogen-bond is rather weak in K and the distance between H$_\zeta$ of the Schiff base and Wat402:O (2.7 Å) is remarkably large compared to other hydrogen-bonds (1.5~1.8 Å), as seen Fig. 7a. Furthermore, during the simulations, the distance between the Schiff base and Wat402 frequently becomes longer due to thermal fluctuation and tension by the strongly deformed chromophore in the Schiff base region. Breaking of the hydrogen-bond between the Schiff base and Wat402 seems to be a prerequisite for the relaxation of the chromophore to a planar conformation in which the N–H bond points “up”, i.e., toward the cytoplasmic side. Although none of the isomerization trajectories applying the Set 1 force field used in the modeling of the K intermediate resulted in
the complete cleavage of this hydrogen-bond, it is likely that the dissociation of the hydrogen-
bond leads to a transition from K to the next intermediate, KL.

Since the lifetime of K can be as long as 100 ns according to a recent TR-FTIR study
(Doiumaev and Braiman, 1997), it is not feasible to observe the decay of K in MD simulations.
Furthermore, the lifetime of K can increase even further due to the strengthened hydrogen-bond
between the Schiff base and Wat402 in the Set 1 force field. Unfortunately, it is rather difficult
to determine empirical potential functions that can quantitatively describe the dissociation of
the hydrogen-bonds that involve strong electronic interactions. In order to accelerate the decay
of the K intermediate, therefore, we employed the Set 2 force field, which does not include
the additional function for the hydrogen-bond (see Sec. 2.2), and repeated the simulations to
examine the dynamics of the photoproducts when this hydrogen-bond can be easily broken.

Starting from the initial configurations taken from the BR equilibrium trajectory at time
intervals of 10 ps, thirty two isomerization trajectories, 80 ps long each, were computed The
additional hydrogen-bond function in the Set 1 force field was turned off at the beginning of
isomerizations. The absence of the strong hydrogen-bond did not affect the dynamics of the
isomerization; all isomerizations took place toward Asp212, as seen in the simulations of the K
intermediate. In addition, the first photoproduct is also similar to the K intermediate obtained
in the simulations with the Set 1 force field, i.e., the chromophore is strongly deformed in the
Schiff base region, and the N–H bond is perpendicular to the membrane normal.

After the formation of the K intermediate characterized as the first photoproduct, however,
about 2/3 of the trajectories (21 in the 32 trajectories) proceeded to a second stable photoproduct in 80 ps. The remaining eleven trajectories stay in K on this time scale. Since the transition is a stochastic process, the eleven trajectories should reach the same second stable state after a longer time.

The excitation energy of the second photoproduct is red-shifted from BR and blue-shifted from K (see below). Therefore, we assign this state to the KL intermediate. Figure 8 shows the QM/MM optimized structure of the KL intermediate. The K-to-KL transition is characterized by significant structural changes in the retinal binding pocket, as illustrated schematically in Fig. 7. The first event is a complete dissociation of the hydrogen-bond between the Schiff base and Wat402, which persists in the K intermediate. The dissociation leads to conformational relaxation of the chromophore to the 13-\textit{cis} planar form, where the N–H bond of the Schiff base points to the cytoplasmic side, and starts to establish a weak hydrogen-bond with Thr89:Oγ. Along with formation of the hydrogen-bond, the Schiff base part of the chromophore becomes twisted again, but in the opposite direction to the K intermediate. One can discern this whiplash motion in Fig. 6c, which displays the retinal binding site in KL, the hydrogen-bond between the Schiff base and Thr89:Oγ, and the twist in the Schiff base region. As will be discussed in Sec. 3.4, the distorted conformation of the Schiff base is stabilized by electrostatic interaction with Asp85, Asp212, and Thr89.

The geometrical parameters of the chromophore in the KL intermediate are listed in Table 3. The $C_{12}-C_{13}-C_{14}-C_{15}$ dihedral angle deviates by 26° from the planar conformation. Although
the C_{14} - C_{15} = N_{\zeta} - C_{\varepsilon} and C_{13} = C_{14} - C_{15} = N_{\zeta} dihedral angles are also twisted considerably, the torsions are somewhat relaxed compared to that in the K intermediate. The relaxed torsions are consistent with the LT- and TR-FTIR and TR-RR measurements, where the 15–HOOP mode in KL ($\sim 980 \text{ cm}^{-1}$) exhibits a high frequency shift from that in the K intermediate ($960 \text{ cm}^{-1}$) (Rothschild et al., 1985; Sasaki et al., 1993; Weidlich and Siebert, 1993; Hage et al., 1996; Dioumaev and Braiman, 1997; Althaus et al., 1995). As previously shown (Tajkhorshid et al., 1999; Tajkhorshid and Suhai, 1999), the H_{15} atom carries a large positive partial charge. The strong hydrogen-bond between H_{15} and Asp212:O_\delta with 2.52 Å distance further stabilizes the twisted conformation in the Schiff base region. This hydrogen-bond can, therefore, also contribute to the aforementioned high frequency shift of the 15–HOOP mode, because the interaction leads to a steeper potential well along a librational motion of the C_{15}–H_{15} bond that corresponds to the HOOP motion.

One also notices in Fig. 6c and 8 a large movement of the Lys216 chain during the K-to-KL transition. In particular, the N_{\zeta}–C_{\varepsilon} single bond rotates by 90.2° (Table 3), and the C_{\varepsilon} atom moves down (Fig. 6c). The conformational change of the chromophore-Lys216 moiety causes a significant rearrangement of the HBN in the binding site. As clearly seen in Fig. 6c, Wat402 is dislocated from its position in the BR and K states (between the Schiff base, Asp85, and Asp212). Due to the complete dissociation of its hydrogen-bond with the Schiff base, and steric interaction with the Lys216:C_{\varepsilon}, Wat402 is forced to move toward the extracellular side, initiating an overall rearrangement of the HBN. Wat401 moves and bridges between Asp85 and
Asp212, and, as a result, the carboxylate group of Asp85 rotates slightly. The dislocation of Wat402 also triggers a downward movement of Arg82 (1.5 Å displacement at Arg82:Cζ) during the K-to-KL transition, as clearly seen in Fig. 8. A remarkable downward shift of the G helix, induced by the conformational change of Lys216, is also observed in the KL structure.

Several structural features discussed above for the KL intermediate are actually consistent with the x-ray crystallography model of K (Edman et al., 1999). The dislocation of Wat402 is in good agreement with the strong negative density in the K–BR difference electron density map. In addition, a significant movement of the Lys216 side-chain around the Cε atom and the downward shift of the G helix are common to both models. Furthermore, the carboxylate group of Asp85 in our KL model is rotated as observed in the x-ray K model, although in our KL model this rotation is smaller and the hydrogen-bond between Thr89 and Asp85 is not broken. Therefore, the x-ray model closely corresponds to the KL intermediate obtained in this study.

The x-ray measurements have been carried out for an intermediate state trapped at 110 K, a temperature 33 K higher than the liquid nitrogen temperature (77 K) used for trapping the K intermediate in spectroscopic studies. This slightly higher temperature might give rise to a mixture of the K and KL states. According to our model, the K formation involves only minor structural changes of the HBN. Therefore, it is likely that the observed difference electron density map is mainly due to structural differences between the BR and KL states. The present models show that the conformations of the chromophore around the Schiff base in K and KL are remarkably different (see Figs. 6b and c, and Table 3). The inhomogeneity due to the
presence of the two K-like species can also account for the absence of a positive density around the Schiff base region of the chromophore in the x-ray difference electron density map (Edman et al., 1999).

The present KL model also indicates the location of Wat402, which has not been identified in the x-ray K model. According to our model, Wat402 moves to a position that closely corresponds to the position of Wat401 in the x-ray model of K. Therefore, the missing water in the x-ray model is the water bridging between Asp85 and Asp212 in our KL model (Wat401 in Fig. 6c). In fact, there seems to be a positive density at this position in the x-ray difference electron density map (see Fig. 5 in Pebay-Peyroula et al., 2000), although no water molecule is modeled at this position in the x-ray structure (PDB code: 1QKO).

The downward movement of Arg82 toward the extracellular side in our KL model is not reported in the x-ray crystallographic study. This disagreement may be due to the different temperatures used for generating the intermediate state. In the x-ray study, the low temperature (110 K) was maintained throughout the experiments, whereas in the present study the KL state was obtained by simulations at room temperature (300 K). Therefore, in the simulations we may observe structural fluctuations that cannot be detected in low temperature experiments. It is noted that the downward movement of Arg82 has been observed in x-ray crystallographic structures of the later states, L (Royant et al., 2000) and M (Luecke et al., 1999b; Sass et al., 2000; Luecke et al., 2000; Facciotti et al., 2001). The movement in the present KL model is, however, much smaller than the motions observed in the x-ray structures of L and M. This
small movement is induced by the HBN rearrangement, especially the downward migration of Wat402, upon the formation of KL.

### 3.4 Energetics of the early intermediates

As seen in the previous sections, the chromophore experiences significant conformational changes during the formation of the early intermediates, resulting in the HBN rearrangement in the retinal binding pocket. Understanding the energetics related to these conformational changes provides an insight into the molecular mechanism of the energy storage in the early phase of the pump cycle. We therefore decomposed and analyzed the QM/MM energies, $E_{\text{QM/MM}}$ (Eqs. 3 and 4), calculated for the early intermediate states. $E_{\text{QM/MM}}$ is defined as the sum of the energies of the QM region itself and its interaction with the MM region. Details of the method are described in Sec 2.4.

Table 4 lists the differences in $E_{\text{QM/MM}}$ between K, KL, and BR. The difference in $E_{\text{QM/MM}}$ between K and BR for System A is 16.2 kcal/mol. Since the MM region in System A stays almost unchanged upon the formation of K (Sec. 3.2), energy of the MM region, $E_{\text{MM}}$, in K is expected to be almost the same as in BR. Thus an enthalpy change, i.e., energy change of the whole system, upon the formation of K can be well described by the energy difference in $E_{\text{QM/MM}}$ alone. The enthalpy change estimated in the present study is in good agreement with the experimental value of 16 kcal/mol (Birge and Cooper; 1983; Birge et al., 1989).

In order to calculate the contribution of the chromophore’s conformational change alone,
we also evaluated $E_{QM/MM}$ for System B, in which only the chromophore is included in the QM region, and Asp85, Asp212, and water molecules of the binding pocket reside in the MM region. The difference in $E_{QM/MM}$ between K and BR for System B is 23.1 kcal/mol, which is 6.9 kcal/mol larger than System A. The difference is due to the slight changes in the structure and electronic interaction of non-retinal groups participating in HBN (Figs. 3 and 7).

Table 4 also summarizes the decomposed contributions to $E_{QM/MM}$ for System B (Sec. 2.4). The difference in $E^0$ is due to the conformational change of the chromophore. The strong torsion around the Schiff base region in K accounts for the large $E^0$ difference between K and BR (11.9 kcal/mol). Change of the chromophore-protein interactions also contributes to $E_{QM/MM}$. The electrostatic stabilization $V_{QM-MM}^{ES}$ in K is significantly smaller (23.3 kcal/mol) than in BR. Upon the formation of K, the H$\zeta$ atom of the Schiff base moves away from such groups as Asp85, Asp212, and Wat402, resulting in the weakening of the electrostatic stabilization. The changes of the electrostatic stabilization effects of Asp85, Asp212, and Wat402 are estimated to be 4.4, 5.6, and 16.5 kcal/mol, respectively. On the other hand, the LJ interaction energy, $V_{QM-MM}^{LJ}$, significantly decreases ($-8.5$ kcal/mol) upon the formation of K. This drastic decrease is mainly due to the reduced strong short-range repulsion between the Schiff base and Wat402 ($-8.8$ kcal/mol). The ERO energy, $E_{QM(MM)}^{ERO}$, also gives a stabilizing contribution ($-3.4$ kcal/mol), consistent with the large destabilizing contribution of $V_{QM-MM}^{ES}$ (Sec. 2.4).

Altogether, upon the formation of K, the energy (16.2 kcal/mol) is stored in the form of the conformational distortion of the chromophore ($E^0 = 11.9$ kcal/mol) and the weakening of
the interaction of the Schiff base with the surrounding polar residues \( (V_{QM-MM}^{ES} + V_{QM-MM}^{LJ} + E_{QM/MM}^{ERO}) = 11.4 \text{ kcal/mol} \), part of which is compensated by the structural and electronic changes of the HBN in the binding pocket \((-6.9 \text{ kcal/mol})\). This scheme is comparable with the results of Birge and Cooper (1983) suggesting that roughly half of the energy is stored in the form of the changes of the electrostatic interaction.

The analysis of energetics for KL is also summarized in Table 4. The difference in \( E_{QM/MM} \) between KL and BR for System A is 29.0 kcal/mol, showing an energy increase by 12.8 kcal/mol from K to KL. This apparent endothermic change is due to the fact that the energy change of the MM region, which experiences conformational changes during the K-to-KL transition, is not included in our analysis; \( E_{QM/MM} \) does not include some of the energy changes caused by the extensive rearrangement of the HBN, e.g., the movement of Arg82 toward the extracellular side in the K-to-KL process (Sec. 3.3). In fact, during the K-to-KL transition, the side chain of Arg82 was found to gain large electrostatic stabilizations from Glu194 \((-10.9 \text{ kcal/mol})\) and Thr205 \((-3.4 \text{ kcal/mol})\) located at the extracellular end of the channel in the MM region, and those energies are not included in \( E_{QM/MM} \). After taking into account such effects, it becomes evident that, despite the apparent increase of \( E_{QM/MM} \) (12.8 kcal/mol), the energy of the KL intermediate is not higher than K.

The increase of \( E_{QM/MM} \) from BR to KL in System B was estimated to be rather small (4.5 kcal/mol) compared to increase from BR to K (Table 4). The increasing stabilization of the chromophore during the K-to-KL transition implies that the energy, which was mainly
stored in the chromophore itself and its interaction with the surroundings in K, is now partially used to rearrange the HBN in the extracellular channel, as seen in Sec. 3.3. The contribution of the conformational distortion of the chromophore, $E^0$, is still large (7.1 kcal/mol) in KL, but 4.8 kcal/mol smaller than K. This is consistent with the relaxed torsion of the Schiff base group in KL, as seen in Table 3. Compared with the K intermediate, the chromophore-protein electrostatic interaction (6.7 kcal/mol) is significantly smaller in KL. Although the hydrogen-bond between the Schiff base and Wat402 completely dissociates during the transition of BR to KL and the chromophore loses large electrostatic stabilization energy (20.8 kcal/mol), the loss is compensated by stabilization effects of Asp85 ($-10.0$ kcal/mol), Thr89 ($-3.4$ kcal/mol), and Asp212 ($-6.7$ kcal/mol). Together with the change of the LJ interaction ($-8.7$ kcal/mol) and ERO ($-0.5$ kcal/mol) contributions, the interaction of the chromophore with its surroundings gives a stabilizing contribution of $-2.6$ kcal/mol.

It is noteworthy that the aforementioned stabilization of Arg82 by Glu194 and Thr205 in the K-to-KL transition accompanies a similar amount of destabilization due to its separation from Asp212 (11.0 kcal/mol) and Asp85 (3.4 kcal/mol), which are main reasons for the observed increase in $E_{QM/MM}$ of System A. Evidently, Arg82 maintains a delicate balance of its interaction with those residues during its movement. Such a balance may account for the conformational flexibility of Arg82.
3.5 Excitation energies

bR possesses photochromic properties and changes its absorption spectrum during the photocycle (Lozier et al., 1975). In the K and KL intermediates, the absorption maxima exhibit red-shifts from the absorption maximum in the BR state. In order to assess the simulated models of the early intermediates, we computed excitation energies of the chromophore by means of the CASSCF QM/MM method (see Sec. 2.5). Table 5 lists the excitation energies for the BR, K, and KL states. The absolute values are largely overestimated compared to the experimental ones, mainly due to the lack of the $\sigma-\pi$ dynamic electronic correlation in the CASSCF description of the ionic $S_1$ state. Nevertheless, the calculations show red-shifts in the maximal absorption of the chromophore upon the formations of the K and KL states, in agreement with the experimental findings (Lozier et al., 1975; Shichida., 1983), although the calculated spectral shifts are too large compared to experimental values.

We have also further analyzed the excitation energies to determine the mechanism of the spectral shifts, and to estimate possible sources of the error in the calculated spectral shifts. The excitation energies, $E_{ex}$, were decomposed into three contributions, $E_{ex}^0$, $\Delta V_{ES}^{ch-pr}$, and $\Delta E_{ERO}^{ch(pr)}$, as described in Sec. 2.5. These contributions are listed in Table 5 for the BR, K, and KL states.

In the K intermediate, $E_{ex}^0$ contributes $-4.1$ kcal/mol to the red-shift relative to BR. This is due to the strongly deformed conformation of the chromophore in the Schiff base region. In particular, strong twists of the $C_{15}=N_\zeta$ and $C_{13}=C_{14}$ double bonds destabilize the highest
occupied π orbital and stabilize the lowest unoccupied π* orbital, leading to a smaller excitation energy. The contribution of $E_{ex}^0$ to the red-shift may be overestimated here, mainly due to a poor description of correlations between the σ and π electrons. The other large contributions come from $\Delta V_{ch-pr}^{ES}$ (−2.2 kcal/mol) and $\Delta E_{ch(pr)}^{ERO}$ (−3.8 kcal/mol). They originate from a large reduction of the electrostatic interaction of the S₀ state with the protein environment during the BR-to-K transition, as discussed in Sec. 3.4 (Table 4). The weaker stabilization of the positive charge results in the red-shift contribution of $\Delta V_{ch-pr}^{ES}$ (Sec. 2.5). At the same time, a weaker interaction gives rise to less polarization of the chromophore in the S₁ state, leading to the red-shift contribution of $\Delta E_{ch(pr)}^{ERO}$.

The red-shift contributions of $\Delta V_{ch-pr}^{ES}$ and $\Delta E_{ch(pr)}^{ERO}$ might be also overestimated in our calculations. The HF method used for the geometry optimization of the chromophore is known to overestimate bonding characters, and, therefore, the torsions in the Schiff base region observed in K are expected to be underestimated. Consequently, the N–H bond tends to point away from the extracellular side too much, resulting in a too weak electrostatic interaction between the Schiff base and Wat402. Our preliminary calculations showed that the distance between Hζ of the Schiff base and Wat402:O can be shorter with only a small energy cost, e.g., 0.7 Å shorter with 3 kcal/mol energy increase, whereas the excitation energy is rather sensitive to this distance.

In the KL intermediate, there also exists a red-shift contribution of $E_{ex}^0$, as seen in Table 5, due to the chromophore torsion in the Schiff base region (see Fig. 6c and Table 3). On the other
hand, the shifts of $\Delta V_{ch-pr}^{ES}$ and $\Delta E_{ch(pr)}^{ERO}$ are somewhat complicated. $\Delta V_{ch-pr}^{ES}$ gives a blue-shift contribution (1.4 kcal/mol), whereas the electrostatic stabilization energy of the $S_0$ state with the surroundings in KL is slightly reduced (6.7 kcal/mol, see Table 4) from that in BR. This stems from a weaker electrostatic stabilization in $S_1$ due to a less polarized electronic wave function. One can see that the counterpart of the $\Delta V_{ch-pr}^{ES}$ contribution arises in $\Delta E_{ch(pr)}^{ERO}$; because of the weaker polarization, destabilization of the $S_1$ state by the electronic reorganization is also smaller. Hence $\Delta E_{ch(pr)}^{ERO}$ provides a red-shift contribution.

The KL intermediate was first introduced by Shichida et al. (1983) based on visible absorption experiments revealing an intermediate that was $\sim 15$ nm blue shifted from K. However, this absorption spectral shift has been questioned by another visible absorption experiment (Yamamoto et al., 1994) where no spectral shift was observed on a nanosecond time scale. The present results support a blue-shift of the spectra in the K-to-KL transition. However, further calculations taking into account electronic correlation (Hayashi and Ohmine, 2000), polarization of the protein matrix (Houjou et al., 2001), and charge-transfer effects will be needed for a definitive understanding of the spectral shift.

4 Discussion

In the present study, we propose structural models for two early intermediate states, K and KL, in bR’s photocycle. The structures of the retinal chromophore and the HBN in the retinal binding site are remarkably different in K and KL. In K the chromophore is strongly twisted in
the Schiff base region and the N–H bond points toward Asp212. The strong torsion in the Schiff base is consistent with LT-FTIR and resonance Raman results, revealing a high intensity peak for the 15–HOOP mode (Pande et al., 1981; Braiman and Mathies, 1982; Bagley et al., 1982; Rothschild and Marrero, 1982). In KL, the chromophore is still twisted, but the N–H bond points toward Asp85 and is hydrogen-bonded to Thr89. The structural changes in the Schiff base region during the K-to-KL transition are in keeping with the characteristic IR spectral change of the 15–HOOP mode observed by LT- and TR-FTIR and TR-RR spectroscopic studies (Rothschild et al, 1985; Sasaki et al., 1993; Weidlich and Siebert, 1993; Hage et al., 1996; Dioumaev and Braiman, 1997; Altaus et al., 1995).

A picosecond TR-RR spectroscopic study (Doig et al., 1991) has reported a large HOOP band in J, the intensity of which decreases significantly after 3 ps, and increases again in 10 to 40 ps. The authors have labeled the HOOP-silent state as K, and the following intermediate as KL, and concluded that the chromophore in the very early intermediate J is highly twisted, but it conformationally relaxes to a more planar form in K, and again undergoes torsions in KL. Since the time scale of the K-to-KL transition in the TR-RR measurement (10 to 40 ps) is completely different from that in the TR-FTIR one (≈100 ns), K in TR-RR is considered to be a new state (K_p), different from K in TR-FTIR, and KL in TR-RR corresponds to K in TR-FTIR (Dioumaev and Braiman, 1997).

In the present study, no planar conformation of the chromophore corresponding to the K_p state was observed. However, it is possible that even a strongly twisted chromophore exhibits a
weak IR intensity of a HOOP mode in $K_p$ due to thermal fluctuation. After the isomerization, the chromophore stores a large amount of excess energy that leads to vibrational excitations. In the short time of $J$ formation, only torsional modes of the polyene skeleton that are directly coupled to the isomerization are vibrationally excited. Then, the excess vibrational energy in the torsional modes is transferred to the HOOP modes by intramolecular vibrational energy redistribution during the $K_p$ formation. The highly excited vibrations of the HOOP modes can give rise to inhomogeneous broadenings of signals, possibly appearing as low intensity peaks. The intensities can recover through the vibrational cooling during the $K_p$-to-$K$ transition.

The topology of the HBN in the vicinity of the Schiff base in $K$ remains the same as in BR. Contrary to the x-ray crystallographic $K$ model (Edman et al., 1999), no dislocation of Wat402 from the position between the Schiff base, Asp85, and Asp212 was observed in our $K$ model. In the $K$-to-$KL$ transition, on the other hand, remarkable hydrogen-bond rearrangements take place. Wat402 is dislocated due to the complete dissociation of its hydrogen-bond with the Schiff base and its steric interaction with Lys216:$\epsilon$. The resulting KL structure corresponds closely to the $K$ intermediate found by the x-ray study (Edman, et al., 1999).

LT-FTIR experiments of Kandori et al. (1998b; 1999; 2001a) have revealed that the hydrogen-bond between Thr89 and Asp85 is stronger in $K$ than in BR, in contradiction to the $K$ model by the x-ray crystallography (Edman et al., 1999), where the carboxylate group of Asp85 largely rotates and the hydrogen-bond is completely broken. The present $K$ and KL models can reconcile the apparent discrepancy; the low temperature vibrational spectroscopies
observe K, whereas the x-ray crystallographic experiment actually observes KL.

A central question is how the early intermediates utilize the photon energy, initially stored through the electronic excitation of the chromophore, to enable bR to pump a proton during the relaxation processes. The present K model clearly indicates that the energy is stored in the form of chromophore’s conformational deformation and weakened interactions between the Schiff base and the surrounding polar side chains and water molecules. In the K-to-KL transition, the chromophore partially relaxes its conformation and establishes stronger electrostatic interactions with the surroundings. However, the conformational relaxation of the chromophore also triggers the rearrangement of the HBN in the binding pocket. Wat402 is pushed out to a less polar region, and loses strong hydrogen-bonds to the negatively charged Asp85 and Asp212. The energy gain of the conformational relaxation of the chromophore is, therefore, utilized to produce a less stable hydrogen-bond configuration. It is noteworthy that the loss of a hydrogen-bond between water and Asp85 can drastically affect the energy profile of the proton transfer between the Schiff base and Asp85, as shown previously by QM/MM calculations (Hayashi and Ohmine, 2000). Hence the rearrangement of the HBN in retinal’s binding site is a key step in the primary proton transfer process of bR.

Since the HBN spans all regions of the extracellular half of the channel, the unstable water configuration in the binding pocket is expected to lead to overall conformational changes in this half (Edman et al., 1999). In fact, a downward displacement of Arg82, a participant of the HBN, was observed in the present KL model. The downward displacement of Arg82 is
suggested to increase the pK$_a$ of Asp85 and decrease that of Glu204, which is located at the extracellular end of the channel and is responsible for the proton release to the extracellular bulk water (Fig. 8). The unstable water configuration allows Arg82 to be more flexible and accomplish the long range correlation between the primary proton transfer in the middle of the channel and the proton release to bulk water at the extracellular surface in the later L-to-M step.

In the KL state, the N–H bond of the Schiff base still points away from Asp85, which is the acceptor in the primary proton transfer. After the KL state, therefore, further conformational changes of the chromophore are required to establish the proton pathway between the Schiff base and Asp85 (Zhou et al., 1993). NMR studies (Hu et al., 1997; Herzfeld and Tounge, 2000; Hatcher et al., 2002) have suggested significantly twisted double bonds of the chromophore in the L and early M intermediates. Accordingly, the strong torsions around the double bonds appearing in the KL state are expected to persist or develop further in the KL-to-L step.

5 Conclusion

The availability of the high resolution x-ray structures of bR together with the accumulated spectroscopic information has set the stage for us to determine the atomic structural models of the early intermediates of bR’s photocycle. We have performed MD simulations and QM/MM calculations to model the K and KL intermediates. The MD simulations were carried out with a faithful description of water molecules that takes into account electronic multi-body effects.
The intermediate structures obtained by the MD simulations of photoisomerization were refined by ab initio QM/MM geometry optimizations, which provide an accurate description of the electronic effects for the chromophore and the HBN. The excitation energies were also computed to identify the intermediates and to analyze the mechanism underlying their bathochromic shifts.

The present study proposes for the first time structural models for both K and KL intermediates. The models reveal important structural details, such as the chromophore conformation and the position of internal water molecules, which are not clearly observed in the x-ray crystallographic model of K. The simulated intermediates can also explain the prominent spectra of K and KL observed in FTIR and resonance Raman spectra and resolve the apparent disagreement between the x-ray structural and FTIR spectroscopic studies. Our QM/MM vibrational normal mode analyses also assign successfully the FTIR spectra of the HBN (Hayashi, et al., in preparation), and explains the underlying mechanism of the remarkable features of the FTIR spectra.

The present structures shed light on the mechanism of energy storage in the early stages of the bR’s photocycle. In K, the strongly deformed chromophore and the weakened interaction of the Schiff base with the counter-ion groups and water are the means of storing the absorbed energy. During the K-to-KL transition, conformational relaxation of the chromophore induces significant HBN rearrangements that facilitate the proton transfers in the later step.

In future studies, similar approaches combining MD and QM/MM simulations can be used
to elucidate other molecular processes in the photocycle of bR, especially the primary proton transfer in the L-to-M step, where the quantum mechanical treatments are necessary. Also, the applied QM/MM methodology together with an improved description of the HBN permits us to seek a more complete understanding of the ultra-fast excited state dynamics of photoisomerization of the chromophore inside the protein matrix.

Acknowledgment

The authors are grateful to Prof. Kandori for stimulating discussions. The work is supported by the Roy J. Carver Charitable Trust, the National Institutes of Health (PHS5 P41RR05969-04), the National Science Foundation (MCB-9982629), and the Human Frontier Science Program Organization. The authors also acknowledge computer time provided by the Grant NRAC:MCA93S028. The molecular images in this paper were created with the molecular graphics program VMD (Humphrey et al., 1996) and rendered with Raster3D (Merritt and Bacon, 1997).

Appendix

In the Set 1 force field, the potential functions of hydrogen-bond interactions of Wat402 with the Schiff base, Asp85, and Asp212 were augmented by an additional term,

\[ E_{add} = E_{NH-LP} + E_{O\delta-H}. \] (6)
$E_{NH-LP}$ is the interaction energy between N–H of the Schiff base and lone pairs of Wat402, described as

$$E_{NH-LP}(r_{O-H}, \theta_1, \theta_2) = \sum_{i=1,2} E_{tap}(\theta_i) A_{NH-LP} e^{-\alpha_{O-H}^2 (r_{O-H} - r_{O-H}^0)^2} e^{-\alpha_{\theta}^2 \theta_i^2}. \tag{7}$$

The variables, $r_{O-H}$ and $\theta_i$, are schematically shown in Fig. 9. $r_{O-H}$ is a distance between the O atom of Wat402 and the H atom of the Schiff base. $\theta_i$ is the angle between the lone pair orbital and $r_{O-H}$. Directions of the lone pairs are set to be tetrahedral, i.e., the angle between the lone pairs is $109.5^\circ$. $E_{tap}(\theta_i)$ is a tapering function applied to remove the disconnectivity around $\theta_i = 180^\circ$. $E_{O\delta-H}$ describes the short-range interactions between the H atoms of Wat402 and O$_\delta$ of Asp85 and Asp212, as

$$E_{O\delta-H}(r_{O\delta-H,1}, r_{O\delta-H,2}) = \sum_{i=1,2} [A_{O\delta-H} \left( \frac{r_{O\delta-H}^0}{r_{O\delta-H,i}} \right)^{10} + H(-r_{O\delta-H,i} + r_{O\delta-H}^0) B_{O\delta-H}(r_{O\delta-H,i} - r_{O\delta-H}^0)^2]. \tag{8}$$

$r_{O\delta-H,1}$ and $r_{O\delta-H,2}$ are distances between H$_2$ of Wat402 and O$_\delta$ of Asp85, and between H$_1$ of Wat402 and O$_{\delta2}$ of Asp212, respectively. $H(r)$ is the Heavyside function.

The parameters were determined so as to reproduce the QM/MM interaction energies, as described in Sec. 2.2; $r_{O-H}^0 = 1.58 \text{ Å}$, $A_{NH-LP} = -10.7 \text{ kcal/mol}$, $\alpha_{O-H} = 1.14 \text{ Å}$, $\alpha_{\theta} = 60.9^\circ$, $r_{O\delta-H}^0 = 1.62 \text{ Å}$, $A_{O\delta-H} = -3.5 \text{ kcal/mol}$, and $B_{O\delta-H} = 2000 \text{ kcal/mol}\cdot\text{Å}^{-2}$.
References


Table 1: Force field parameters for the C$_{12}$–C$_{13}$=C$_{14}$–C$_{15}$ torsional coordinate of protonated retinal Schiff base in the S$_1$ excited state.$^a$

<table>
<thead>
<tr>
<th>n</th>
<th>$V_n/2$</th>
<th>$\gamma$</th>
<th>n</th>
<th>$V_n/2$</th>
<th>$\gamma$</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>20.26</td>
<td>180.0</td>
<td>5</td>
<td>0.11</td>
<td>180.0</td>
</tr>
<tr>
<td>2</td>
<td>-6.80</td>
<td>0.0</td>
<td>6</td>
<td>-0.04</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>-0.79</td>
<td>180.0</td>
<td>7</td>
<td>-0.02</td>
<td>180.0</td>
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<tr>
<td>4</td>
<td>0.33</td>
<td>0.0</td>
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</tbody>
</table>

$^a$ Potential energy for the torsion is expressed as $\sum_n (V_n/2)(1 + \cos(n\phi_{13=14} - \gamma))$, where $\phi_{13=14}$ is the dihedral angle. $^b$ Values are in kcal/mol. $^c$ Values are in degree.
Table 2: RESP atomic charges\(^a\) of the chromophore in the ground (S\(_0\)) and excited (S\(_1\)) states.

<table>
<thead>
<tr>
<th>atom</th>
<th>S(_0)</th>
<th>S(_1)</th>
<th>atom</th>
<th>S(_0)</th>
<th>S(_1)</th>
<th>atom</th>
<th>S(_0)</th>
<th>S(_1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(_\gamma)</td>
<td>-0.1115</td>
<td>-0.1157</td>
<td>C(_{11})</td>
<td>-0.0439</td>
<td>-0.0704</td>
<td>C(_2)</td>
<td>0.0033</td>
<td>0.0040</td>
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<tr>
<td>H(_\gamma)</td>
<td>0.0279</td>
<td>0.0282</td>
<td>H(_{11})</td>
<td>0.1394</td>
<td>0.0852</td>
<td>H(_2)</td>
<td>0.0086</td>
<td>0.0101</td>
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<tr>
<td>C(_\delta)</td>
<td>0.0178</td>
<td>0.0271</td>
<td>C(_{10})</td>
<td>-0.1435</td>
<td>-0.0816</td>
<td>C(_1)</td>
<td>0.0094</td>
<td>0.0148</td>
</tr>
<tr>
<td>H(_\delta)</td>
<td>0.0256</td>
<td>0.0250</td>
<td>H(_{10})</td>
<td>0.1252</td>
<td>0.1517</td>
<td>C(_{20})</td>
<td>-0.0004</td>
<td>-0.0038</td>
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<tr>
<td>C(_\varepsilon)</td>
<td>0.0407</td>
<td>0.0153</td>
<td>C(_9)</td>
<td>-0.1179</td>
<td>-0.0743</td>
<td>H(_{20})</td>
<td>0.0440</td>
<td>0.0385</td>
</tr>
<tr>
<td>H(_\varepsilon)</td>
<td>0.0665</td>
<td>0.0702</td>
<td>C(_8)</td>
<td>-0.0951</td>
<td>-0.0461</td>
<td>C(_{19})</td>
<td>-0.0006</td>
<td>-0.0008</td>
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<tr>
<td>N(_\zeta)</td>
<td>-0.0601</td>
<td>-0.1453</td>
<td>H(_8)</td>
<td>0.0823</td>
<td>0.0857</td>
<td>H(_{19})</td>
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<td>0.0605</td>
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<td>H(_\zeta)</td>
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<td>0.4031</td>
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<td>-0.0392</td>
<td>C(_{18})</td>
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<td>0.0059</td>
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<tr>
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<td>H(_{15})</td>
<td>0.2646</td>
<td>0.2401</td>
<td>C(_6)</td>
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<td>-0.0011</td>
<td>C(_{17})</td>
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<td>-0.0172</td>
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<tr>
<td>C(_{14})</td>
<td>-0.1780</td>
<td>-0.1618</td>
<td>C(_5)</td>
<td>0.0260</td>
<td>0.0757</td>
<td>H(_{17})</td>
<td>-0.0005</td>
<td>0.0006</td>
</tr>
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<td>H(_{14})</td>
<td>0.1401</td>
<td>0.1625</td>
<td>C(_4)</td>
<td>0.0031</td>
<td>0.0061</td>
<td>C(_{16})</td>
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<td>C(_{13})</td>
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<td>-0.0595</td>
<td>H(_4)</td>
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<td>0.0230</td>
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<td>0.0014</td>
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<td>C(_{12})</td>
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<td>0.0167</td>
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<td>H(_{12})</td>
<td>0.1869</td>
<td>0.2009</td>
<td>H(_3)</td>
<td>-0.0089</td>
<td>-0.0115</td>
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</tbody>
</table>

\(^a\) Restrained electrostatic potential derived charges (Bayly et al., 1993).
Table 3: Geometrical parameters of the chromophore in the BR, K, and KL states. Angles are in degree.

<table>
<thead>
<tr>
<th>Bond Configuration</th>
<th>BR</th>
<th>K</th>
<th>KL</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₅=Nₛ–C₆–C₇</td>
<td>-112.8</td>
<td>-149.3</td>
<td>120.6</td>
</tr>
<tr>
<td>C₁₄=C₁₅=Nₛ–C₆</td>
<td>-166.8</td>
<td>149.2</td>
<td>-157.2</td>
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<tr>
<td>C₁₃=C₁₄–C₁₅=Nₛ</td>
<td>-178.2</td>
<td>162.2</td>
<td>-171.4</td>
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<tr>
<td>C₁₂=C₁₃=C₁₄–C₁₅</td>
<td>-161.3</td>
<td>-25.4</td>
<td>26.4</td>
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<td>C₁₁=C₁₂–C₁₃=C₁₄</td>
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<td>170.6</td>
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<td>C₁₀=C₁₁=C₁₂–C₁₃</td>
<td>-172.7</td>
<td>168.3</td>
<td>-168.0</td>
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<td>C₉=C₁₀–C₁₁=C₁₂</td>
<td>-175.0</td>
<td>-172.6</td>
<td>-179.5</td>
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<tr>
<td>C₈=C₉=C₁₀–C₁₁</td>
<td>-178.0</td>
<td>-171.3</td>
<td>-176.0</td>
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<tr>
<td>C₇=C₈–C₉=C₁₀</td>
<td>-172.6</td>
<td>-172.9</td>
<td>-176.1</td>
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<tr>
<td>C₆=C₇=C₈–C₉</td>
<td>176.9</td>
<td>174.6</td>
<td>177.8</td>
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<tr>
<td>C₅=C₆–C₇=C₈</td>
<td>169.7</td>
<td>168.0</td>
<td>177.1</td>
</tr>
<tr>
<td>φᴺᴴᵃ</td>
<td>19.8</td>
<td>82.3</td>
<td>236.1</td>
</tr>
<tr>
<td>φᵥ²₀ᵇ</td>
<td>158.9</td>
<td>128.7</td>
<td>155.3</td>
</tr>
</tbody>
</table>

ᵃ Angle of the N–H bond of the Schiff base to the membrane normal. ᵇ Angle of the C₁₃–C₂₀ bond to the membrane normal.
Table 4: Changes of QM/MM energies ($E_{QM/MM}^a$) upon the formation of K and KL, and their additive contributions$^b$, $E^0$, $V_{QM-MM}^{ES}$$^d$, $V_{QM-MM}^{LJ}$$^e$, $V_{QM-MM}^{BAD}$$^f$, and $E_{QM(MM)}^{ERO}$$^g$ in kcal/mol.

<table>
<thead>
<tr>
<th></th>
<th>$E_{QM/MM}$</th>
<th>$E^0$</th>
<th>$V_{QM-MM}^{ES}$</th>
<th>$V_{QM-MM}^{LJ}$</th>
<th>$V_{QM-MM}^{BAD}$</th>
<th>$E_{QM(MM)}^{ERO}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>K–BR</td>
<td>16.2$^h$,  23.1$^i$</td>
<td>11.9</td>
<td>23.3</td>
<td>−8.5</td>
<td>−0.2</td>
<td>−3.4</td>
</tr>
<tr>
<td>KL–BR</td>
<td>29.0$^h$,  4.5$^i$</td>
<td>7.1</td>
<td>6.7</td>
<td>−8.7</td>
<td>0.1</td>
<td>−0.5</td>
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</table>

$^a$ A sum of the energy of the QM region and the QM-MM interaction. The energies were evaluated for two QM/MM system, System A (the QM region consists of retinal-Lys216, Asp85, Asp212, Wat401, Wat402, and Wat406) and System B (the QM region consists of retinal-Lys216). $^b$ Additive energies were computed for System B. $^c$ QM energy in isolated condition. $^d$ Electrostatic interaction between the QM and MM region. $^e$ Lennard-Jones interaction between the QM and MM region. $^f$ Bond, angle, and dihedral energies at the QM-MM boundary. $^g$ Electronic reorganization energy. $^h$ Results for System A. $^i$ Results for System B.
Table 5: Excitation energies ($E_{ex}$) of the retinal chromophore in the BR, K, and KL states, and contributions to the excitation energy, excitation energy of the isolated chromophore ($E_{ex}^0$), electrostatic interaction between chromophore and protein matrix ($\Delta V_{ch-pr}^{ES}$), and electronic reorganization energy of the chromophore ($\Delta E_{ch(pr)}^{ERO}$) (all energies are in kcal/mol).

<table>
<thead>
<tr>
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<th>$E_{ex}$</th>
<th>$E_{ex}^0$</th>
<th>$\Delta V_{ch-pr}^{ES}$</th>
<th>$\Delta E_{ch(pr)}^{ERO}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR</td>
<td>98.3 (50.3$^b$)</td>
<td>68.3</td>
<td>23.3</td>
<td>6.7</td>
</tr>
<tr>
<td>K$^a$</td>
<td>-10.1 (-3.5$^c$)</td>
<td>-4.1</td>
<td>-2.2</td>
<td>-3.8</td>
</tr>
<tr>
<td>KL$^a$</td>
<td>-3.5 (-1.8$^b$)</td>
<td>-3.2</td>
<td>1.4</td>
<td>-1.6</td>
</tr>
</tbody>
</table>

$^a$ Values are relative to BR. $^b$ Absorption maxima at room temperature (Lozier et al., 1975). $^c$ Absorption maxima at 77 K (Birge et al., 1989).
Legend to Figures

Figure 1. Schematic representation of all-trans retinal bound via a protonated Schiff base to Lys216 in bR, and its conventional atom numbering.

Figure 2. Comparison of the x-ray crystallographic structures in the vicinity of the Schiff base in (a) BR (Luecke et al., 1999, PDB: 1C3W) and (b) K (Edman et al., 1999, PDB: 1QKO) states. The β-ionone halves of the chromophore are not shown. In the structure of the K intermediate, dislocation of Wat402 and rotation of the carboxyl group of Asp85 are observed. Wat400 in the K model of Edman et al. (1999) corresponds to Wat406 in the model of BR of Luecke et al. (1999)

Figure 3. Schematic representation of the hydrogen-bond network in the binding pocket in the BR state, and atom numbering used in the text. Dashed lines indicates possible hydrogen-bonds. Distances of the hydrogen-bonds are shown in Å.

Figure 4. Energy profile of the potential function of the C₁₃=C₁₄ dihedral coordinate, φ₁₃=₁₄, of the retinal chromophore used in the MD simulations of the photoisomerization. In the electronical ground state, the dihedral potential energy function possesses a minimum at the 13-trans configuration, represented by a dashed line. At the time photoabsorption takes place, the potential function is switched to the reactive one, represented by a solid line, which leads to the isomerization to the 13-cis configuration. There are two possible directions of the isomerization, rotation of the N–H bond of the Schiff base toward Asp85 or toward Asp212, since the
dihedral potential function is symmetric. The vertical energy difference of the dihedral potential functions at the planar 13-\textit{trans} configuration is set to be 39.1 kcal/mol, which together with the contribution from the chromophore-protein electrostatic interaction, $\Delta V_{ch-pr}^{ES} = 11.2$ kcal/mol, reproduces the experimental absorption energy (50.3 kcal/mol).

\textbf{Figure 5}. Comparison of the interaction energies of Wat402 with its surroundings, obtained by different empirical force field functions, with those obtained by means of the QM/MM method. The energies were evaluated at 27 conformations that were generated by changing orientation of the Wat402 molecule from the energy minimum geometry. $\times$ and $\square$ indicate results of the TIP3P and Set 1 models (Sec. 2.2), respectively.

\textbf{Figure 6}. Structural changes in the vicinity of the Schiff base in the early phase of the photocycle, modeled in the present study. Stereo views are shown for the QM/MM optimized structures of (a) BR, (b) K, and (c) KL. The hydrogen-bonds are depicted as dashed lines. Wat402 in the calculated KL structure corresponds closely to Wat401 in the x-ray structure model of K (Edman et al., 1999), and Wat401 in the present model is suggested to be the missing water in the x-ray model (Sec. 3.3).

\textbf{Figure 7}. Schematic representations of the hydrogen-bond network (HBN) in the binding pocket in (a) K and (b) KL. Dashed thin lines represent hydrogen-bonds. Distances of the hydrogen-bonds are shown in Å. Dashed thick arrows in gray illustrate conformational changes of the hydrogen-bond network during the K-to-KL transition.
**Figure 8.** Structures of the K and KL intermediates. The seven transmembrane helices are shown as cylinders; loops are shown in tube representation; β-strands are drawn as ribbons. The retinal chromophore and key amino acids are highlighted (K is shown in gray, blue, and red; KL is shown in green). Water molecules (W401, W402, and W406) are represented as red (K) and green (KL) spheres.

**Figure 9.** Schematic representation of geometrical parameters used in the augmenting potential describing the hydrogen-bond interactions between Wat402 and its neighbors.
Figure 1:
Figure 2:
Figure 3:
Figure 4:
Figure 5:
Figure 6:
Figure 7:
protonated Schiff base
Wat402
$q^q$
1
2
$r_{O-H\zeta}$
$\theta_1$
$\theta_2$

Figure 9: