Molecular Dynamics Study of the Nature and Origin of Retinal's Twisted Structure in Bacteriorhodopsin

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ABSTRACT The planarity of the polyene chain of the retinal chromophore in bacteriorhodopsin is studied using molecular dynamics simulation techniques and applying different force-field parameters and starting crystal structures. The largest deviations from a planar structure are observed for the C_{13}═C_{14} and C_{15}═N_{16} double bonds in the retinal Schiff base structure. The other dihedral angles along the polyene chain of the chromophore, although having lower torsional barriers in some cases, do not significantly deviate from the planar structure. The results of the simulations of different mutants of the pigment show that, among the studied amino acids of the binding pocket, the side chain of Trp-86 has the largest impact on the planarity of retinal, and the mutation of this amino acid to alanine leads to chromophore planarity. Deletion of the methyl C_{20}, removal of a water molecule hydrogen-bonded to H_{15}, or mutation of other amino acids to alanine did not show any significant influence on the distortion of the chromophore. The results from the present study suggest the importance of the bulky residue of Trp-86 in the isomerization process, in both ground and excited states of the chromophore, and in fine-tuning of the pK_a of the retinal protonated Schiff base in bacteriorhodopsin. The dark adaptation of the pigment and the last step of the bacteriorhodopsin photocycle imply low barriers against the rotation of the double bonds in the Schiff base region. The twisted double bonds found in the present study are consistent with the proposed mechanism of these ground state isomerization events.

INTRODUCTION

The transmembrane protein bacteriorhodopsin (bR) present in the purple membrane of *Halobacterium salinarium* (formerly *H. halobium*) is one of the simplest known active membrane transport systems (Oesterhelt and Stoeckenius, 1973; Oesterhelt, 1976; Henderson, 1977). It functions as a light-driven proton pump converting light energy into a proton gradient which is used by the cell as an energy source to produce ATP. Structurally, it folds into seven transmembrane helices, one of them containing the residue Lys-216 at which the retinal prosthetic group binds via a protonated Schiff base linkage. The chromophore divides the channel formed by the α-helices of the polypeptide into a cytoplasmic part connecting to the inside of the cell and an extracellular part connecting to the outside (Oesterhelt et al., 1992; Mathies et al., 1991; Lanyi, 1993; Rotschild, 1992; Krebs and Khorana, 1993; Schulten et al., 1995).

The general features of the transport mechanism are now understood. Absorption of a photon, which starts the bR photocycle, induces an excited state of the chromophore followed by the isomerization of the retinal protonated Schiff base around the C_{13}═C_{14} bond (the double bond next to the Schiff base group). During the next thermally activated steps of the bR photocycle, the initially protonated retinal Schiff base releases a proton into the extracellular part of the channel and will be reprotonated again from a proton source located in the cytoplasmic part. Therefore, a proton is effectively pumped from the inside of the cell to the outside during each cycle. Finally, the chromophore will be isomerized back to the all-*trans* form in the last step of the photocycle (Oesterhelt et al., 1992; Mathies et al., 1991; Lanyi, 1993; Rotschild, 1992; Krebs and Khorana, 1993).

The proton pump mechanism starts by a proton transfer from the protonated retinal Schiff base to the next proton-accepting group that is suggested to be the negatively charged carboxylate group of Asp-85 in the protein backbone (Lanyi, 1993). Evidence is accumulating about a possible involvement of at least one water molecule in this step. The involvement of water molecules in the stability of the protonated Schiff base was suggested by DuPuis et al. (1980), and by Hildebrandt and Stockburger (1984) on the basis of the resonance Raman study of dried membranes. The presence of water in the binding site was shown by de Groot et al. (1989) on the basis of the ^{15}N-NMR studies. Recent crystal structures of bR also demonstrate the presence of a few water molecules in the vicinity of the Schiff base group (Pebay-Peyroula et al., 1997; Luecke et al., 1998). The effect of the water molecules on the pK_a of the Schiff base group is demonstrated by pK_a measurement of a series of retinal Schiff base analogs (Gat and Sheves, 1993). The possible positions of hydrogen-bonded water molecules around the Schiff base group have been theoretically examined (Nina et al., 1995; Roux et al., 1996). The structure, dynamics, and energetics of bR have been studied by molecular dynamics simulations of the all-*trans* ground state of bR (Ferrand et al., 1993; Humphrey et al., 1995; Xu et al., 1995, 1996; Roux et al., 1996), and of the dark-adapted state

Received for publication 14 June 1999 and in final form 28 October 1999.
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0006-3495/00/02/683/11 $2.00

(Logunov et al., 1995; Logunov and Schulten, 1996; Baudry et al., 1999). Retinal photoisomerization in bR has also been investigated using QM/MM potentials (Warshel et al., 1991) or quantum dynamics of the whole protein (Ben-nun et al., 1998), as well as by free energy simulations (Hermone and Kuczera, 1998).

The transport mechanism is based on the sequential changes in the pK_a values of the retinal Schiff base and vectorially arranged protonatable groups in the protein. The pK_a change of respective groups in the proton channel, especially the pK_a of the retinal Schiff base, plays a crucial role in the proton transfer reaction. There are several possible reasons explaining why the pK_a of the Schiff base would be lowered at the beginning of deprotonation. Among these are the disruption of the π-system of the retinal Schiff base chain during the *trans*-to-*cis* isomerization, which decreases the electronic density of the Schiff base nitrogen (Orlandi and Schulten, 1979; Tavan et al., 1985), and the conformational changes that modify the electrostatic environment of the retinal Schiff base (Warshel and Levitt, 1976; Warshel, 1979, 1986; Tajkhorshid and Suhai, 1999a, b) or change the orientation of the hydrogen-bonded groups (Scheiner and Hillenbrand, 1985; Scheiner and Duan, 1991).

The decrease in the pK_a of the Schiff base is the first step that may induce the proton transfer. It should be mentioned, however, that the pK_a of the retinal Schiff base significantly increases in the protein environment compared with its isolated form. It is known from experimental data that the pK_a of the protonated retinal Schiff base in methanol/water (1:1) solution is ~7.2 (Baasov and Sheves, 1986; Rousso et al., 1995), while the pK_a in bR is shifted to 13.3 (Druckman et al., 1982; Sheves et al., 1986). The protein environment seems to have a very strong effect on the pK_a of the retinal Schiff base. The presence of the negatively charged side chains of Asp-85 and Asp-212 in the vicinity of the protonated Schiff base is proposed to have the main influence on the electronic structure and charge distribution of the retinal Schiff base in the bR protein environment (Tajkhorshid and Suhai, 1999c).

According to the available crystallographic data for retinal (Simmons et al., 1981; Hamanaka et al., 1972) and N-methyl-N-phenylimine retinal Schiff base (Santarsiero et al., 1990), the retinal polyene molecule has a planar structure in its isolated form. The strong steric hindrance of the substituted methyl groups on the polyene chain is, to some extent, compensated by adoption of a banana-shaped structure by the molecule. In the protein environment, however, the steric interaction of the binding pocket with the chromophore may strongly influence the planarity of the chromophore. A twisted retinal structure in the bR protein environment is supported by experimental results. The biphasic nature of the CD spectrum of the retinal Schiff base in bR has been explained on the basis of the adoption of a twisted structure by the chromophore (Wu and El-Sayed, 1991). In a recent paper, more evidence for nonplanarity of the retinal Schiff base structure in the protein environment has been reported after the analysis of the optical rotation of the second harmonic radiation from retinal in bR monomers in Langmuir-Blodgett films (Volkov et al., 1997). Polarized infrared spectroscopy studies also suggest the existence of distortions around different dihedral angles along the retinal chain (Weidlich and Siebert, 1993; Weidlich et al., 1996).

Experimental NMR studies indicate the possibility of the isomerization of the C_{15}═N_{16} and C_{13}═C_{14} double bonds in the ground state for different analogs of the protonated retinal Schiff base in solution (Sheves and Baasov, 1984; Albeck et al., 1992). It has also been reported that the protonated retinal Schiff base chromophore is able to perform ground state rotation around the C_{13}═C_{14} (and C_{15}═N_{16}) double bond(s) in the protein environment. This is, for instance, the case during the last step of the bR photocycle (rotation around the C_{13}═C_{14} bond). This is also the case for dark-adaptation of bR. Dark-adaptation occurs via a double isomerization of the C_{13}═C_{14} and C_{15}═N_{16} bonds. Both experimental (Balashov et al., 1995, 1996) and theoretical (Logunov et al., 1996) results suggest that a transient protonation of Asp-85 during the dark-adaptation lowers the rotational barrier of these bonds. The C_{13}═C_{14} and C_{15}═N_{16} double bonds can then be co-isomerized, probably following the "bicycle-pedal" pathway (Baudry et al., 1999).

Theoretical calculations have also shown that these two double bonds have the lowest isomerization barriers among the double bonds of the main polymer chain in the protonated Schiff base model (Tajkhorshid et al., 1999). Accordingly, because of the low barrier against the rotation of these double bonds, the retinal structure may acquire a twisted form around one or both of these double bonds in the protein environment. If this happens, a part of the pK_a increase of the retinal Schiff base in bR, as compared to the solution form of the chromophore, can be related to the protein-induced twist in the double bonds in the vicinity of the Schiff base group (Orlandi and Schulten, 1979; Tajkhorshid et al., 1999).

Importance of the planarity of the retinal Schiff base

The structure of all-*trans* protonated retinal Schiff base and its conventional atom numbering are depicted in Fig. 1. Conjugated Schiff base molecules such as the retinal Schiff base possess a delocalized π-electronic system. Even in the unprotonated species, because of the larger electronegativity of the nitrogen atom, the conjugated π-electron system is slightly shifted toward the Schiff base group (C═N). In the protonated species, because of the additional positive charge on the protonated Schiff base group, the delocalization is much more pronounced. In both protonated and unprotonated species, the delocalization is mainly observed

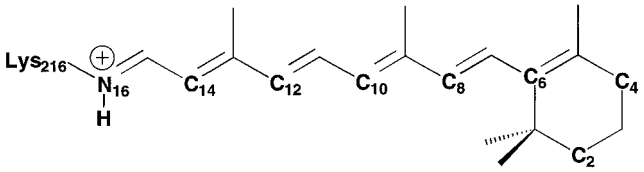

FIGURE 1 The protonated retinal Schiff base in its all-trans geometry and its conventional atom numbering.

in the Schiff base region. The extent of the π-electron delocalization determines the bond distances and the isomerization barriers against the rotation of different single or double bonds along the chain, which in turn influence the planarity of the chromophore.

Dihedral angles are important structural aspects of the conjugated Schiff base structures. The planarity of the main chain of the polyene is necessary for the maximum conjugation of the double bonds. Particularly in the protonated species, this is essential for the compensating effect of the conjugated double bonds on the positive charge of the Schiff base group (C═N), and subsequently, on the pK_a of the molecule (Tajkhorshid et al., 1997). Any strong steric interaction between the substituted groups on the polyene chain (for example, steric interaction between the substituted methyl groups and the adjacent hydrogen atoms) or between the retinal and the protein environment may induce a twisted structure in the chromophore which, in turn, significantly influences the pK_a of the Schiff base (Orlandi and Schulten, 1979; Tajkhorshid et al., 1997, 1999).

Theoretical calculations suggest that the rotation around a single bond results in a decrease of the pK_a of the chromophore (Orlandi and Schulten, 1979; Tajkhorshid et al., 1997, 1999). This decrease is related to the disruption of the conjugation effect along the polyene chain during the rotation of a single bond (Orlandi and Schulten, 1979; Tajkhorshid et al., 1999). Rotation around a double bond in the Schiff base terminus of the molecule (C_{13}═C_{14} and/or C_{15}═N_{16}), however, was surprisingly predicted to have a different effect on the pK_a of the molecule (Tajkhorshid et al., 1999). In the protonated form of a conjugated Schiff base molecule, the rotation around these double bonds causes the transfer of the positive charge from the Schiff base region to the other terminus of the molecule. In the transition state of these rotations the protonated Schiff base group converts to a secondary amine group. This will decrease the extent of the positive charge on the nitrogen atom and, therefore, increase the pK_a of the molecule during the rotation around the double bonds (Tajkhorshid et al., 1999).

Crystallographic studies have shown that the retinal molecule and the protonated retinylidene Schiff base molecule adopt a "banana-shaped" structure (Simmons et al., 1981; Hamanaka et al., 1972; Santarsiero et al., 1990). This can be explained, on the one hand, by the large steric interaction of the substituted methyl groups on the main chain of the polyene structure and, on the other hand, by the significant barriers against the rotation of the conventional single bonds (Tajkhorshid et al., 1999), which could otherwise compensate for such steric interactions. Therefore, because the polyene cannot easily rotate around the single bonds, the methyl steric interactions have to be partly compensated for by the adoption of the known banana-shaped backbone, and not a twisted structure.

Theoretical calculations also predict a planar structure for the retinal Schiff base molecule (Tajkhorshid et al., 1997; Tajkhorshid and Suhai, 1999d). According to the gas-phase ab initio calculations, all of the dihedral angles of the main polyene chain are 180° ± 1.5°, with the exception of the C_5═C_6 and C_6—C_7 bonds, which deviate from the planar structure by 7.5° and 9.0°, respectively (Tajkhorshid et al., 1997; Tajkhorshid and Suhai, 1999d). The C_5═C_6 and C_6—C_7 bonds are located in the β-ionone ring region, where large intramolecular steric interactions are present. It has to be mentioned that a significantly more twisted structure in this region can be found for the 6s-*cis* conformer of the retinal, i.e., the form found in solution. This is mainly because of the stronger steric interaction between the methyl group on C5 and the hydrogen atoms of the polyene chain, in the 6s-*cis* conformer. In bR, however, a ring-chain s-*trans* planar conformation of the retinal chromophore has been experimentally observed rather than the twisted 6s-*cis* conformation observed in solution. The planarity of ring-chain in bR was first suggested by Schreckenbach et al. (1978). The s-*trans* conformation of the chromophore in bR was deduced from ^{13}C-NMR chemical shifts (Harbison et al., 1985). X-ray crystallographic studies (Santarsiero et al., 1990) also suggest a planar ring-chain structure for the all-*trans* conformer of the retinal Schiff base.

The large steric interaction between retinal's methyl groups and hydrogens can be partially compensated by the adoption of a banana-shaped structure for the chromophore. Despite this compensation, the structural strains originating from the methyl groups result in a destabilization of the planar structure. Consequently, the presence of methyl groups leads to a reduction of rotational barriers around covalent bonds in the chromophore.

We examine the retinal structure in the binding pocket of bR to explore whether and how the steric interaction with the protein environment may influence the chromophore planarity. Analysis of such interactions at the atomic level provide more information about the possible mechanism(s) by which the protein significantly decreases the isomerization barriers against the rotation of the C_{13}═C_{14} and C_{15}═N_{16} double bonds and changes the pK_a of the chromophore.

Molecular dynamics simulations of wild-type and mutated bR structures have been performed to study the structure of the retinal Schiff base in the presence of the protein environment of bR. To further examine how the protein environment may influence the planarity of the retinal chro-

mophore, different mutations of bR were also studied. The main focus of the paper will be on the dihedral angles of the main polyene chain of the chromophore as a measure of the planarity of the chromophore in the binding pocket.

COMPUTATIONAL METHODS

The CHARMM (Brooks et al., 1983) potential energy function was used in all the molecular dynamics simulations and geometry optimizations of bR. The potential function has the following form:

$$\begin{aligned} V = & \sum_{\text{bonds}} k_b (b - b_o)^2 + \sum_{\text{angles}} k_\theta (\theta - \theta_o)^2 \\ & + \sum_{1:3} k_u (u - u_o)^2 + \sum_{\text{dihedrals}} k_\phi (1 + \cos[n\phi - \delta]) \\ & + \sum_{\text{impropers}} k_\omega (\omega - \omega_0)^2 \\ & + \sum_{i,j} 4\varepsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{6} \right] \\ & + \sum_{i,j} \frac{1}{4\pi\varepsilon} \frac{q_i q_j}{r_{ij}} \end{aligned} \quad (1)$$

The potential energy function includes bonded interactions (bond stretches, bond angles, and dihedral angle contributions), and nonbonded interactions between pairs (i, j) of atoms. In Eq. 1, b, u, θ, and ω are the bond lengths, Urey-Bradley 1:3 distances, bond angles, and improper dihedral angles in any given configuration, and b_0, u_0, θ_0, and ω_0 are the reference values for these properties; the associated force constants are k_b, k_u, k_θ, and k_ω. The improper dihedral contributions are used to represent out-of-plane deformations of the sp2 groups. For the intrinsic dihedral angles ϕ, k_ϕ is the force constant, n is the symmetry number of the rotor (e.g., 3 for a methyl group), and δ is the phase angle.

The nonbonded interactions are included between pairs i, j of atoms separated by three or more bonds. They consist of a Lennard-Jones term, with parameters ϵ_{ij} and σ_{ij} and a Coulombic electrostatic term between partial charges q_i, q_j. The dielectric constant, $\epsilon = \epsilon_0 \times \epsilon_r$ was set to $\epsilon = \epsilon_0$, i.e., $\epsilon_r = 1$. Hydrogen bonds are described by the nonbonded terms in the energy function. In all the calculations long-range electrostatic terms were smoothly brought to zero at a cutoff of 12 Å by multiplication by a cubic switching function between 10 and 12 Å. Pairs of atoms on the same molecule separated by only two bonds may interact via a Urey-Bradley term harmonic in the distance between atoms i, j.

Two different sets of parameters were used for the retinal part during the simulations. This allows a comparison of the effect of the applied parameters on the obtained results with respect to the chromophore planarity. In the first parameter set for retinal (retinal parameter set A), parameters given in Baudry et al. (1997) were used. These parameters allow a correct reproduction of semi-empirical calculations of rotations around double bonds (Humphrey et al., 1995), of ab initio calculations of water-retinal interaction (Nina et al., 1995) and of (13, 15)-*dicis*/all-*trans* experimental ratio in dark-adapted bR (Baudry et al., 1999).

In the second retinal parameter set (retinal parameter set B) the atomic charges and equilibrium bond distances for the retinal Schiff base were extracted from ab initio calculations (Tajkhorshid et al., 1997; Tajkhorshid and Suhai, 1999d), where density functional calculations were performed on the complete structure of the protonated retinal Schiff base using GAUSSIAN 94 (Frisch et al., 1985). The hybrid Becke3LYP method and 6–31G** basis set were used for the DFT calculations. All of the stationary points were confirmed to be the minimum by calculation of analytical second derivatives (Tajkhorshid and Suhai, 1999d). The charges were derived from a Mulliken population analysis. For the force constants of the dihedral angles of the main polyene chain of the retinal Schiff base we used the barriers reported for a model Schiff base with the same number of the double bonds. These barriers are calculated using the unrestricted Becke3LYP/6–31G* SCF level of theory (Tajkhorshid et al., 1999). The dihedral parameters used for the retinal in sets A and B are summarized in Tables 1 and 2, respectively.

Two different crystallographic structures were used as the starting geometry of bR in the calculations. One structure (structure S1) is from Pebay-Peyroula et al. (1997), (PDB entry 1AP9). The other starting structure (structure S2) is

TABLE 1 The parameter set A for the torsional potentials of the retinal in CHARMM notation

ϕ_I	k_i (kcal/mol)†	n_i	δ_i (deg)
C_X═C_X—C_X═C_X*	0.1600	1	0.0
	2.2318	2	180.0
	1.1324	3	0.0
	0.3006	4	0.0
X—C_7═C_8—X*	5.8750	2	180.0
X—C_9═C_{10}—X*	4.1500	2	180.0
X—C_{11}═C_{12}—X*	3.5500	2	180.0
C_{13}═C_{14}—C_{15}═N_{16}*	1.504	1	180.0
	5.3010	2	180.0
	0.9160	3	0.0
	0.4010	4	0.0
X—C_{13}═C_{14}—X*†	3.1500	2	180.0
C_{12}—C_{13}═C_{14}—C_{15}‡	0.6500	1	0.0
X—C_{15}═N_{16}—X*†	3.5500	2	180.0
C_{14}—C_{15}═N_{16}—C_ε‡	0.5100	1	180.0

"X" denotes an arbitrary atom. For rotations around double bonds, four terms contribute to the $n = 2$ dihedrals, and one term to the $n = 1$ dihedrals. For rotations around single bonds, only carbon atoms of the polyene main chain are included in the contribution to the $n = 1, 2, 3, 4$ dihedrals, except for the C_{13}═C_{14}—C_{15}═N_{16}, where the nitrogen of the Schiff base is included in the contribution of the $n = 1, 2, 3, 4$ dihedrals.
*Nina et al., 1993, 1995.
†Humphrey et al., 1995.
‡Baudry et al., 1997, 1999.

TABLE 2 The parameter set B used for the torsional potentials of the main polyene chain of the retinal Schiff base

ϕ_i	k_i (kcal/mol)*	n_i	δ_i (deg)
$C_5{=}C_6{-}C_7{=}C_8$	11.24	2.0	180.00
$C_6{-}C_7{=}C_8{-}C_9$	39.98	2.0	180.00
$C_7{=}C_8{-}C_9{=}C_{10}$	17.03	2.0	180.00
$C_8{-}C_9{=}C_{10}{-}C_{11}$	37.28	2.0	180.00
$C_9{=}C_{10}{-}C_{11}{=}C_{12}$	22.50	2.0	180.00
$C_{10}{-}C_{11}{=}C_{12}{-}C_{13}$	35.08	2.0	180.00
$C_{11}{=}C_{12}{-}C_{13}{=}C_{14}$	28.30	2.0	180.00
$C_{12}{-}C_{13}{=}C_{14}{-}C_{15}$	29.46	2.0	180.00
$C_{13}{=}C_{14}{-}C_{15}{=}N_{16}$	30.43	2.0	180.00
$C_{14}{-}C_{15}{=}N_{16}{-}C_{\varepsilon}$	28.76	2.0	180.00

Tajkhorshid et al., 1999.
*$E_i^{\text{dihedral}} = (1/2)k_i[1 + \cos(n_i\varphi_i - \delta_i)]$.

from Luecke et al. (1998), (PDB entry 1BRX). A similar initial placement of water molecules was used to equilibrate each structure. The details of the calculations and of the equilibration of the structures will be published later, but here we give the results of our work on the hydration of bR within a hydrogen-bond distance of retinal. To place water molecules in the retinal binding pocket of structure S1, a free energy perturbation theory was used following the protocol of Roux et al. (1996). Within a hydrogen-bond distance of retinal, two possible hydration sites were found to have a non-zero probability of occupation by a water molecule.

One position for a water molecule, labeled WA in Fig. 2, is in excellent agreement with the position of water "W402" in the structure of Luecke et al. (1998). It is located in the extracellular channel and forms a bifurcated hydrogen bond with the Schiff base and residues Asp-85 and Asp-212. Our free energy calculations indicated a probability of existence close to 1 for a water molecule at this position in the protein. The other water molecule, labeled WB in Fig. 2, was calculated to have a lower probability of existence in the protein of ~0.7. This water molecule is located in the cytoplasmic half of the proton channel and makes a hydrogen bond with the hydrogen atom located on C_{15} in retinal. This position is close to a position proposed by Roux et al. (1996) using the structure of Grigorieff et al. (1996) with a significantly lower probability in our calculations. This position and low thermodynamic stability are in agreement with the position and B factor of water molecule labeled "6" in the crystal structure of Pebay-Peyroula et al. (1997), that was defined by the authors of the crystal structure as not well-resolved. As the thermodynamic stability of water molecule WB is low in our calculations and possibly in Pebay-Peyroula et al.'s (1997) crystal structure as well, we will investigate the effect of its deletion from the model on the retinal planarity. The crystal structure S2 (Luecke et al., 1998) was hydrated by replicating in S2 the positions of the water molecules placed in the retinal's binding site of model S1.

The hydrated wild-type S1 and S2 structures were equilibrated using Langevin molecular dynamics simulations performed at 300 K with an integration time step of 2 fs. The total equilibration time was 75 ps, with average values calculated from the last 37 ps of equilibration. For the protein part of bR, version 22 of the CHARMM force field was used (MacKerell et al., 1998). For the retinal part, the retinal parameter set A (Table 1) was used in the first stage of the study.

In the second stage, retinal parameter set B (Table 2) was used for both wild-type and mutant forms of bR. An additional 21 ps of equilibration were run starting from the average structure of Pebay-Peyroula et al. (structure S1) obtained from the first stage of the study. Calculation of

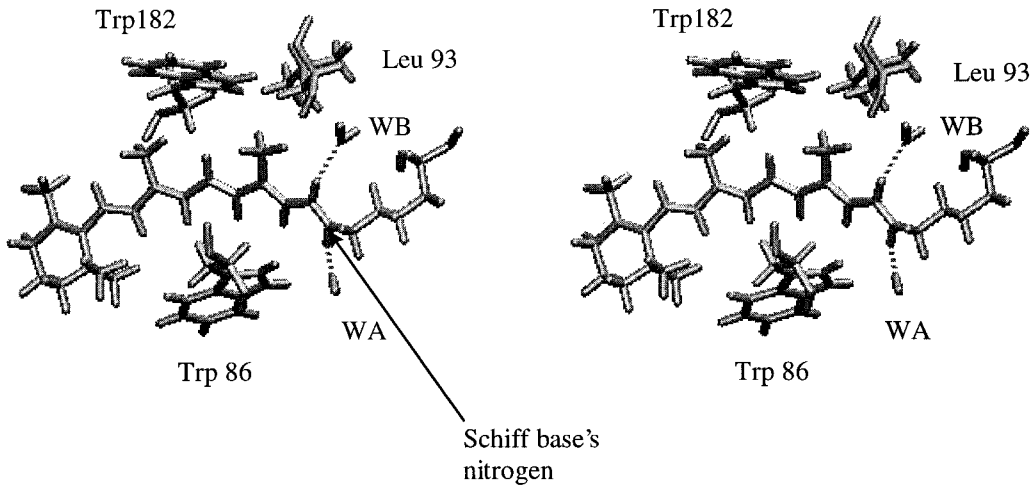

FIGURE 2 Stereo view of the retinal binding pocket of bR obtained from the minimized average structure of the MD simulation of the wild-type pigment. Only the amino acids selected as candidates for further modification and exploration of the specific steric interaction of the chromophore and protein are shown, as well as the two water molecules discussed in the text.

average values of the retinal dihedral angles was done using the last 18 ps of simulations.

The average structures were energy-minimized using 500 steps of steepest descent followed by 1000 steps of Adapted Basis Newton-Raphson optimization algorithms. During all equilibrations and energy minimization runs the backbone of bR was restrained around the initial crystal structure by applying harmonic restraints of 2 kcal/mol/Å^2 on the backbone atoms further than 18 Å from the Schiff base nitrogen atom, so as to maintain the shape of the protein during simulations at 300 K (Ferrand et al., 1993).

The application of different starting geometries and different force-field parameters permits a comparison of the significance of the applied force-field parameters and the influence of the initial bR structures on the final results. To investigate the influence of the protein residues and water molecules present in the binding pocket on the retinal planarity, five modifications of structure S1 were equilibrated using force field set B:

- A bR in which Leu-93 is mutated to Ala (L93A);
- A bR in which Trp-86 is mutated to Ala (W86A);
- A bR in which Trp-182 is mutated to Ala (W182A);
- A bR in which the methyl group C_{20} is replaced by hydrogen (called "13-demethyl-retinal");
- A bR in which water molecule WB (Fig. 2) (close to the hydrogen H_{15} of retinal in the cytoplasmic channel) is deleted (called dehydrated H_{15}).

RESULTS AND DISCUSSION

Nonplanarity of the chromophore in bR

The calculated dihedral angles for the retinal chromophore for the average structures S1 and S2, with their standard deviations, and for the energy-minimized structures of S1 and S2 are compiled in Table 3. These angles can be used to define the planarity of the chromophore in the binding pocket. Table 3 shows that in all cases the dihedral angles of the average structure and the corresponding values for the minimized average structure are quite close to each other. This suggests an essentially harmonic dynamical behavior of the dihedral angles under study here, under the conditions of simulations. The average values of the dihedral angles obtained from the simulations applying structures S1 or S2 are very close, with differences being of the order of 5°. In particular, the nonplanar structure of retinal in the Schiff base region (see below) is found using both starting structures.

In the wild-type form of the pigment retinal was found to be not completely planar; for two of the dihedral angles deviations of ~15° from the planar structure can be seen. These dihedral angles correspond to the C_{13}═C_{14} and C_{15}═N_{16} conventional double bonds and not to the single bonds. As can be seen in Table 3, all of the single bonds, while having smaller torsional forces in most cases, were found to be almost planar during the MD simulations.

Examination of the dihedral force constants reveals that, except for C_{14}—C_{15} and C_{12}—C_{13} single bonds whose rotation barriers are comparable with the double bonds, other single bonds have significantly lower isomerization barriers. In the Schiff base region of the chromophore we can distinguish four bonds that possess very close isomerization barriers; the isomerization barriers around the C_{15}═N_{16}, C_{14}—C_{15}, C_{13}═$C_{14,}$ and C_{12}—C_{13} bonds are calculated to be in the range 28.30–30.43 kcal/mol. Therefore, there is only a maximum difference of ~2.0 kcal/mol between the isomerization barriers of the last four bonds in the polyene backbone of the retinal Schiff base. However, a twist is only seen for the C_{15}═N_{16} and C_{13}═C_{14} double bonds. Specific steric interactions between the chromophore and the binding pocket may be responsible for this behavior. Strains originating from such interactions could then be relaxed through the rotation of the (conventional) double bonds and not the single bonds in the Schiff base region.

The force constants of the single bond or double bond rotation change along the polyene chain. As we get closer to

TABLE 3 Dihedral angles (degree) of the main polyene chain of retinal Schiff base calculated for the average (av) and the minimized average (min-av) structures of the chromophore during the MD simulation in the protein environment

	Structure S1, retinal parameter set A			Structure S2, retinal parameter set A			Structure S1, retinal parameter set B	
Species	av	SD	min-av	av	SD	min-av	av	min-av
N_{16}═C_{15}	−166.6	6.9	−164.9	−170.1	6.6	−170.5	−168.2	−166.9
C_{15}—C_{14}	172.2	8.7	170.8	−178.6	8.5	−173.6	176.5	176.2
C_{14}═C_{13}	−165.5	7.7	−164.6	−166.3	6.8	−165.2	−167.6	−166.2
C_{13}—C_{12}	173.9	10.0	173.7	178.1	9.8	177.7	178.5	178.8
C_{12}═C_{11}	−171.2	6.7	−170.0	−176.7	6.0	−178.1	−173.2	−172.1
C_{11}—C_{10}	−175.6	11.0	−176.2	−171.7	9.6	−170.1	−178.6	−177.3
C_{10}═C_{9}	−175.3	6.0	−174.3	−179.3	6.2	179.7	−176.1	−175.1
C_{9}—C_{8}	171.1	10.9	173.7	−178.9	10.1	−179.6	178.3	177.9
C_{8}═C_{7}	179.9	5.6	−179.4	177.7	5.3	177.4	179.9	−179.8
C_{7}—C_{6}	−156.7	12.9	−159.2	175.3	10.8	176.3	−168.9	−168.7

Standard deviations (SD) are also shown for the first two sets of MD simulations.

the Schiff base region, a single bond rotation becomes more difficult, whereas the rotation around a double bond becomes energetically more affordable as compared with the region close to the β-ionone ring. Examination of the standard deviations of the average values in the case of simulation of wild-type structures show that the "double" or "single" character of the bond is preserved, though. The standard deviation for the conventional double bonds is always smaller than for the conventional single bonds. The standard deviations of the average values show that although the C_{13}═C_{14} and C_{15}═N_{16} dihedral angles are distorted, their movements remain more restrained around their average values than the single bonds C_{12}—C_{13} or C_{14}—C_{15}, whereas these single bonds have a more planar average structure.

It has been shown that the isomerization of either the C_{13}═C_{14} or the C_{15}═N_{16} double bonds in the protonated polyene Schiff base facilitates the rotation of the other double bond (Orlandi and Schulten, 1979). Therefore, the deviation of one of these double bonds from a planar structure results in a decrease of the barrier of rotation of the other double bond. For instance, the rotation around the C_{13}═C_{14} double bond has been shown to convert the double bond of the Schiff base group (C_{15}═N_{16}) to a single bond at the transition state of the rotation (Tajkhorshid et al., 1999). The barriers in Tables 1 and 2 can thus be considered an overestimate of the dihedral force constants for the rotation of the double bonds. Accordingly, a larger deviation from the planarity could be expected for the double bonds of the Schiff base region (C_{15}═N_{16} and C_{13}═C_{14}). This cooperative effect is not taken into account in the present classical simulations. This effect can be investigated using full-quantum description of the nuclei's dynamics (Ben-Nun et al., 1998) or QM/MM approaches (Warshel et al., 1991).

The recent bR crystal structures used as starting points for our calculations (Pebay-Peyroula et al., 1997, PDB entry 1AP9; Luecke et al., 1998, PDB entry 1BRX) reported strongly twisted dihedral angles around the C_{15}═N_{16} double bond. In these structures the C_{15}═N_{16} double bond deviates by ~48.0 and 33.0° from a planar structure (180.0°), respectively. The C_{13}═C_{14} torsional angle was, however, found to be planar in these crystal structures, whereas our calculations suggest that a retinal twisted around this C_{13}═C_{14} bond can be found in bR. This suggestion is in very good agreement with a more recent crystal structures for the bR trimer that gives values for the C_{13}═C_{14} torsional angle comprised between −163.4 and −165.2°; and values for the C_{15}═N_{16} torsional angle comprised between −162.0 and −164.3 (Essen et al., 1998, PDB entry 1BRR). A very recent structure also indicates twisted retinal around the C_{13}═C_{14} and C_{15}═N_{16} torsional angles, with values for these angles of −156.9 and −162.7°, respectively (Luecke et al., 1999).

The deviation of the double bonds close to the Schiff base region of the retinal chromophore from a planar structure results in an increase of the electron density on the nitrogen atom of the Schiff base group (Tajkhorshid et al., 1999). Therefore, the pK_a of the retinal chromophore can be increased by a twist around the C_{13}═C_{14} and C_{15}═N_{16} double bonds. Retinal performs ground state rotations around the C═N and C_{13}═C_{14} double bonds in the protein environment during the dark-adaptation of the pigment in a time scale of several minutes, and rotation around the C_{13}═C_{14} double bond during the final steps of the photocycle in a time scale of several microseconds. The predicted change of proton affinity is consistent with the point that, during the ground state rotations of these double bonds, no proton transfer from the chromophore to the environment takes place.

The twisted structure found for the retinal chromophore in the present study, therefore, can be responsible for at least a part of the pK_a increase of the retinal after binding to bR apoprotein. This conclusion is in agreement with the lowered pK_a values experimentally measured for the "locked" all-*trans* retinal analogs (Rousso et al., 1995). For instance, the pK_a value of the Schiff base group in artificial bR pigment with a locked chromophore was measured to be 11.5, whereas the pK_a in native bR is 13.3. In the locked chromophore species the twist and/or the rotation around the C_{13}═C_{14} double bond is hindered through tailoring a cyclic structure, impeaching the subsequent pK_a increase in these analogs. According to the results of the present study and previous theoretical calculations (Tajkhorshid et al., 1999), an even lower pK_a value would be expected if one could, in addition, block the twist of the C═N group by means of chemical modifications.

In conclusion, we suggest that in addition to the retinal/protein electrostatic interactions, steric effects between the chromophore and the protein-binding pocket are important means for the fine-tuning of the retinal's pK_a.

The significance of the dihedral force constants

The extent of the π-electron delocalization in a polyene structure can be estimated by the examination of the bond alternation in the main chain. For example, examination of the bond distances of the different conventional single and double bonds along the main chain and the deviation of these bonds from the pure single or double bonds, respectively, shows that in a protonated polyene Schiff base, the bond alternation is significantly decreased in the Schiff base region (Orlandi and Schulten, 1979; Tavan et al., 1985; Tajkhorshid et al., 1997, 1999; Tajkhorshid and Suhai, 1999c, 1999d).

The bond alternation of the main chain directly influences the bond order and, consequently, the isomerization barrier of different single and double bonds. However, depending on the applied model, the extent of the π-electron delocal-

ization can be different (Paizs et al., 1999). For example, it is reported that DFT calculations overestimate the π-electron delocalization in the retinal molecule (Bifone et al., 1996), as compared with the experimental structures (Simmons et al., 1981; Hamanaka et al., 1972). Comparison of the DFT-optimized structures of a series of conjugated Schiff base models with those obtained from CAS-SCF calculations also show the trend of DFT to underestimate bond alternation in conjugated Schiff base molecules for both protonated and unprotonated species (Paizs et al., 1999). For example, after full optimization of the retinal Schiff base geometry at the B3LYP/6–31G* level of theory (Tajkhorshid et al., 1997; Tajkhorshid and Suhai, 1999d), the C_{15}═N_{16} and C_{13}═C_{14} bonds are predicted to be ~0.056 Å longer than the experimental values (Santarsiero et al., 1990). This difference is much smaller for the single bonds. The C_{15}—C_{14} and C_{13}—C_{12} single bonds are predicted to be 0.010 and 0.016 Å shorter than experimental bond distances, respectively. It has to be mentioned that the deviations of the DFT results from the available experimental structure of the retinal N-methyl-N-phenyliminium perchlorate (Santarsiero et al., 1990) can be partly related to the presence of a negatively charged ion in the vicinity of the Schiff base group in the crystal structure. This negative charge, which causes the bond alternation of the polyene to be partially recovered (Tavan et al., 1985), is absent in the DFT calculations mentioned above.

As stated in the previous section, the molecular dynamics simulations indicated that the protein chromophore steric interaction is very efficient and spatially oriented in a way that induces a twisted structure around the double bonds. To examine the effect of the above-mentioned methodological problems in the calculation of the dihedral force constants, we manipulated the applied torsional parameters in several ways and repeated the MD simulations. In most of the cases the modifications of the dihedral force constants included a downscaling of the single bond rotation barriers and/or an increase of the isomerization barrier against the double bond rotations. This gives the chromophore a chance to relax its structure by the rotation around a single bond rather than around a double bond. Furthermore, the possibly underestimated bond alternation of the retinal Schiff base by DFT during the calculation of the isomerization barriers can also be corrected in this way. However, it turned out that in none of the cases did the scaling of the dihedral force constants influence the conclusions about the importance of the double bond rotations for the compensation of the steric interaction of the protein and the chromophore.

Analysis of the simulated bR mutants

Application of different dihedral force constants did not exhibit a significant effect on the position of the twist in the retinal chromophore. This suggests that a specific steric interaction of the chromophore with the binding pocket is responsible for the nonplanarity of the chromophore, rather than intramolecular interactions in the retinal. This suggests further exploration of the protein-chromophore interaction. To examine different possible origins of steric interactions between retinal and the surrounding protein environment, we repeated the MD simulations for the bR mutants and modified pigments listed in Methods.

The residues Trp-86, Leu-93, and Trp-182, water molecule WB (Fig. 2), and the methyl substitution on the C_{13} atom of the chromophore were modified. For the point mutations, an alanine residue replaced the amino acid side chain. The effect of the water molecule and of the C_{13} methyl group was studied by deletion of the water molecule from the coordinate file and replacement of the methyl by a hydrogen atom, respectively. The dihedral angles obtained for molecular dynamics simulations using these mutants and modified retinal are given in Table 4. With the exception of the W86A mutation, none of the mutations or modifications could recover the planarity of the chromophore in the binding pocket of bR.

Calculations did not show any significant effect of the substituted methyl group on C_{13} on the dihedral angles

TABLE 4 Dihedral angles (degree) of the main polyene chain of retinal Schiff base calculated for the average (av) and the minimized average (min-av) structures of the chromophore during the MD simulation of different modified species of bR

	W86A		W182A		L93A		13-demethyl-retinal		Dehydrated H_{15}	
Species	av	min-av	av	min-av	av	min-av	av	min-av	av	min-av
N_{16}═C_{15}	−175.8	−176.2	−166.6	−165.9	−167.8	−168.9	−166.8	−167.1	−166.5	−166.8
C_{15}—C_{14}	177.4	177.3	175.5	174.6	176.2	177.4	176.0	176.8	176.5	176.7
C_{14}═C_{13}	−178.0	−176.4	−166.4	−165.8	−166.3	−166.8	−164.7	−163.3	−164.6	−164.7
C_{13}—C_{12}	178.5	177.9	178.7	178.7	178.6	178.5	178.3	177.8	178.4	178.6
C_{12}═C_{11}	−176.6	−175.6	−172.6	−172.7	−171.6	−171.2	−170.6	−168.8	−172.0	−171.7
C_{11}—C_{10}	−179.9	179.9	−176.8	−176.4	−178.4	−179.5	−177.8	−178.6	−178.3	−177.8
C_{10}═C_{9}	−176.9	−176.2	−174.9	−175.1	−174.7	−174.0	−173.8	−172.6	−175.0	−175.2
C_{9}—C_{8}	176.5	176.6	177.6	177.6	177.5	175.5	177.4	176.7	177.7	177.9
C_{8}═C_{7}	−179.8	179.1	−179.1	179.3	−178.7	−178.4	−178.8	−178.9	−179.9	178.9
C_{7}—C_{6}	−171.2	−169.4	−168.5	−168.5	−169.2	−171.4	−168.7	−168.3	−167.9	−166.8

For all simulations the retinal parameter set B has been used.

(Table 4). However, this paper concentrates only on the planarity of the chromophore in the binding pocket. It has been shown that the methyl substitutions are important structural aspects of the retinal Schiff base (Tajkhorshid and Suhai, 1999d). The methyl groups can also be important for the conformational coupling of the protein and the chromophore in different steps of the photocycle. The location of the methyl groups on the polyene side chain is of the utmost importance in determining the overall shape of the retinal ligands (de Lera et al., 1995). These structural effects, added to the dominant steric and electronic restrictions of the binding pocket (Logunov et al., 1996a; Song et al., 1996) would explain the discrimination exhibited by the retinal-binding site for different analogs during incubation studies (Logunov et al., 1996a). These effects can also influence the rate of the photoisomerization and dynamics of the ground and excited states of the retinal Schiff base (Logunov et al., 1996a, b; Song et al., 1993, 1996). Mutations of Leu-93 to Ala did not show a significant effect on the dihedral angles of the all-*trans* retinal, even though interactions between Leu-93 and retinal are involved in the rapid thermal reisomerization of retinal in bR's photocycle (Delaney et al., 1997). Deletion of water molecule WB did not show a significant effect on retinal's dihedral angles, either.

After mutation of the bulky side chain of Trp-86 to alanine, the C_{13}═C_{14} and C_{15}═N_{16} dihedral angles became significantly closer to the planar values. Examination of the MD trajectory of wild-type bR suggest that the indole group of Trp-86 is spatially interacting with the polyene chain in the Schiff base region, and the local conformational changes of this group are strongly coupled to the chromophore structural changes. This role of tryptophan is also suggested in other retinal binding proteins. In human red opsin, for example, the importance of the tryptophan residues in the proper folding of the protein and the retinal-protein interaction have been recently reported (Nakayama et al., 1998). The presence of four tryptophan residues in the chromophore binding pocket of bR may also be considered as an indication of the importance of tryptophan side chains in the construction of the binding pocket in retinal proteins.

CONCLUSIONS

In this paper the structure of the retinal chromophore in the binding pocket of bacteriorhodopsin (bR) has been studied and the potential effects of the protein environment on the structure of the chromophore have been explored. Because of the relatively low barriers against the rotation of the C_{13}═C_{14} and C_{15}═N_{16} double bonds, and the large steric interaction of the chromophore with its protein environment, a twisted structure around these double bonds and/or the C_{14}—C_{15} single bond can be proposed for the retinal.

The results suggest that the steric interactions in the binding pocket of bR can be compensated only by rotation around the double bonds, rather than around single bonds. For the wild bR, the largest deviations from a planar structure can be seen for the C_{13}═C_{14} and C_{15}═N_{16} double bonds in the Schiff base region. The deviation of the double bonds from the planar structure was found to be mainly originating from the specific arrangement of the amino acids in the binding pocket and is not significantly dependent on the applied force field. These results are consistent with the fact that the chromophore is able to undertake ground-state isomerization around these double bonds in the last step of the bR photocycle as well as in the dark adaptation process. Furthermore, at least a part of the pK_a increase of the retinal in the bR binding pocket can be related to the deviations of the C_{13}═C_{14} and C_{15}═N_{16} double bonds from a planar structure.

To further explore the details of the steric interactions between the chromophore and the binding pocket of bR, several modifications of retinal or its immediate environment in the bR binding pocket were also examined. Among the studied groups in the retinal binding pocket, Trp-86 was found to play the main role in imposing the nonplanarity of the chromophore. This suggests an important role of this residue in the coupling of the conformational changes of the protein and the chromophore, which may also influence the pK_a of retinal as the central part of the proton transfer path.

The authors thank Prof. Eva Pebay-Peyroula and Prof. Hartmut Luecke for providing us with their crystal structures. We thank Dr. Ferenc Molnar for help with calculations and fruitful discussions, and Prof. Mordechai Sheves for valuable discussions during the course of the study. We also thank Prof. Benoit Roux, Prof. Jeremy Smith, Dr. Mafalda Nina, and Dr. Regis Pomes for helpful discussions in the course of water placement in bR models. K.S. thanks the Institute of Advanced Studies of the Hebrew University in Jerusalem for its hospitality.

J.B. and K.S. acknowledge support from the National Institute of Health (Grant PHS 5P41 RR05969), the National Science Foundation (Grant NSF BIR 94-23827EQ, NSF/GCAG BIR 93-18159, MCA93S028), and the Roy J. Carver Charitable Trust.

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