The energy-storage, the electronic-excitation energy-shift and the molecular rearrangements due to the primary photochemical event in rhodopsin are investigated using QM/MM (MO:MM) hybrid methods in conjunction with high-resolution structural data of bovine visual rhodopsin. It is assumed that the primary process involves 11-cis/all-trans isomerization of the retinyl chromophore within a time scale much shorter than the protein relaxation time. The analysis of the molecular structures, obtained after electronic- and partial nuclear-relaxation along the 11-cis/all-trans isomerization path, reveals the detailed molecular rearrangements of the retinyl chromophore in the rhodopsin binding pocket; the preferential sense of rotation of the φ(C11-C12) dihedral angle; and the detailed chromophore-protein interactions responsible for the energy-storage mechanism and the initial rotational torque. It is revealed that the φ(C11-C12) dihedral angle changes from -10° in the 11-cis isomer to -165° in the all-trans isomer, with a preferential sense of rotation determined by the steric Van der Waals interactions between Ala117 and the polyene chain at the C13 position. The polyene chain of the all-trans isomer is significantly bent and twisted due to space constraints in the binding pocket, including out-of-the-plane distortions larger than 15° in the dihedral angles of the C7-C8, C9-C10, C12-C13, C14-C15 and C15-NH(+) chromophore bonds. It is shown that the energy-storage computed at the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory is 29 kcal/mol, in very good agreement with experimental measurements (32±3 kcal/mol). It is predicted that 40% of the energy stored is strain energy, due to the steric constraints of the all-trans isomer in the rhodopsin binding pocket. The remaining 60% is electrostatic energy due to stretching of the salt-bridge between the protonated Schiff-base and the Glu113 counterion. The analysis of the salt-bridge stretching mechanism indicates that the underlying process involves solely torsion of the polyene chain. The consequent reorientation of the polarized bonds C15-H and N-H+ displaces the net positive charge at the Schiff-linkage, relative to the Glu113 counterion, without displacing the linkage itself, or inducing a redistribution of atomic charges within the chromophore. It is demonstrated that a hydrogen-bonded water molecule, consistently found by X-ray crystallographic studies, can assist the salt-bridge stretching process by stabilizing the reorientation of the polarized bonds at the Schiff-linkage. Finally, it is shown that the ONIOM-EE (TD-B3LYP/6-31G*:B3LYP/6-31G*:Amber) level of theory predicts that the S0 → S1 electronic-excitation energy-shift caused by the underlying molecular rearrangements is ΔΔE = 3.2 kcal/mol, in quantitative agreement with experimental measurements (3.4 kcal/mol). These results are particularly relevant to the development of a first principles understanding of the structure-function relationship of G-protein-coupled receptors at the detailed molecular level.

I. INTRODUCTION

Understanding the structure-function relationship of G-protein-coupled receptors (GPCRs) at the detailed molecular level is a subject of great interest, since GPCRs are the central components of a variety of biological signal transmission pathways\textsuperscript{1–3}. The membrane glycoprotein rhodopsin is a prototypical GPCR present in the rod cells of the retina\textsuperscript{4–5}, responsible for turning on the signaling transmission cascade in the vertebrate vision process\textsuperscript{6–8}. In this paper, quantum mechanics/molecular mechanics (QM/MM) hybrid methods are implemented to investigate the structure-function relationship of rhodopsin in terms of an analysis of the molecular rearrangements due to the primary photochemical event and the influence of the protein environment on the reaction energetics.

The primary photochemical event in vision involves absorption of visible light by rhodopsin and its conversion to the metastable intermediate bathorhodopsin, shown in Fig. 1. Rhodopsin is composed of a bundle of seven transmembrane α-helices surrounding the covalently bound retinyl chromophore. The chromophore 11-cis-rhodopsin isomerizes to the all-trans isomer during the primary photochemical event\textsuperscript{9–11}. The formation of bathorhodopsin is endothermic and stores approximately 50% of the photo-energy\textsuperscript{12–15}. The stored energy is subsequently used to promote thermal reactions in the protein bleaching sequence and in the subsequent transducin cycle.

The detailed molecular mechanism responsible for the storage of the photon-energy through photo-isomerization of the retinyl chromophore in rhodopsin has been the subject of intense research and remains an open problem\textsuperscript{16–25}. The reaction is complete within 200 ns.
fs with a high quantum-yield (\(\sim 0.67\)) that is wavelength-dependent \(^{26,27}\) making it one of the fastest and most efficient photo-reactions in nature \(^{1-3}\). Intriguingly, however, the isomerization of the 11-cis retinal in solutions is much slower (e.g., involves a 10 ps reaction time in methanol \(^{28}\) ) and has a much lower efficiency (e.g., quantum yield \(\sim 0.2\), independent of wavelength in at least four different solvents \(^{29}\) ). Furthermore, the wavelength of maximum absorbance of the retinyl chromophore in rhodopsin is significantly shifted relative to the absorption of retinal in solutions and approximately coincides with the wavelength of maximum intensity of the solar spectrum (\(\sim 500\) nm). These findings suggest that the molecular environment in rhodopsin has been highly optimized by natural selection for absorption of visible light and energy-storage through photo-isomerization of the retinyl chromophore. Progress in understanding the effect of the environment on the reaction speed and efficiency has been partially hindered for many years by the lack of high-resolution structural data.

Theoretical studies of the primary photochemical event in rhodopsin have a long history \(^{30-35}\). Most of them were performed much before the crystallographic structure of rhodopsin was available. The pioneer studies by Warshel and co-workers \(^{31-33}\) were based on the semi-empirical QCFF/PI method for the description of the chromophore and a representation of the protein-chromophore interaction based on a surface of closed-packed spheres described by a model potential with adjustable parameters. More recently, the QCFF/PI surfaces have been re-calibrated \(^{36}\) to reproduce the trend of \(ab\ initio\) studies for the isolated chromophore \(^{37,38}\) but applied only in studies of bacteriorhodopsin \(^{36}\). Birge and co-workers \(^{34,35}\) have implemented MNDO/AM1 and INDO-PSCDI procedures and have described the photo-isomerization dynamics in terms of a one-dimensional potential model with an arbitrary rate constant for the dissipation of the internal energy. These early theoretical studies proposed energy-storage mechanisms based on a mixture of charge separation and protein strain \(^{33,39}\) as alternatives to the existing charge separation models \(^{12,40}\). However, rigorous calculations of the strain and electrostatic contributions were not possible since the protein structure was not known.

The recently reported X-ray crystal structure of bovine rhodopsin \(^{41,42}\) is the first high-resolution structure of a GPCR. This structure, although at a resolution of 2.8 Å, offers an opportunity to understand the photo-isomerization mechanism in rhodopsin at the detailed molecular level and to elucidate the influence of the protein environment on the energetics, speed and efficiency of the reaction. The X-ray crystal structure of bovine rhodopsin has already motivated theoretical studies that focused on the analysis of the geometry and electronic excitation energy of the \(S_0 \rightarrow S_1\) transition of the retinyl chromophore, including Density-Functional-Theory (DFT) calculations based on the self-consistent-charge tight-binding approximation \(^{22,43}\), classical molecular dynamics simulations \(^{19}\), \(ab\ initio\) Restricted Hartree Fock (RHF) calculations of reduced-model systems \(^{44}\) and quantum mechanics/molecular mechanics computations at the CASPT2//CASSCF/Amber level of theory \(^{18}\). In particular, the \(ab\ initio\) studies provided a rigorous description of the chromophore in the dark state. However, the detailed conformational changes responsible for the storage of energy through 11-cis/all-trans isomerization still remain largely elusive to first principles examinations.

The formation of the photo-product bathorhodopsin within 200 fs after photoexcitation of the system indicates that the detailed microscopic dynamics involves ultrafast electronic-relaxation to the ground electronic state on vibronically coupled potential energy surfaces. Recent quantum calculations have explored the underlying curve crossing process \(^{37,38,45-49,49,50}\). These rigorous calculations, however, have not included an explicit description of the binding pocket environment due to the complexity of the problem.

In this paper, two-layer QM/MM(MO:MM) hybrid methods are implemented to describe the retinyl chromophore rearrangements due to the 11-cis/all-trans isomerization, after electronic relaxation to the ground state subject to the constraint of a fixed protein environment. This study thus includes an explicit treatment of the protein environment and assumes that such environment remains mostly unchanged during the ultrafast 200 fs reaction time. This assumption is subsequently validated by comparing the predicted energy storage and spectroscopic energy shift to experimental data. The ONIOM-EE (B3LYP/6-31G*:Amber/6-31G*/TD-B3LYP:Amber) levels of theory are implemented to investigate the electronic-excitation energy-shift and the energy-storage mechanism due to conformational changes of the retinyl chromophore. The ONIOM hybrid approach \(^{31-56}\), as implemented in the Gaussian package \(^{57}\), has been extensively tested against the standard Complete-Active-Space Self-Consistent-Field (CASSCF) method. It has been demonstrated that ONIOM can accurately reproduce the standard CASSCF(10e/10o) (i.e., 10 active electrons in 10 orbitals) results for the first singlet excited state (\(S_1\)) photoisomerization pathways in protonated Schiff-bases \(^{58}\). In addition, the two-layer QM:MM combination implemented in this paper has been successfully applied by Vreven and Morokuma in a recent study of the \(S_0 \rightarrow S_1\) vertical excitation in bacteriorhodopsin \(^{59}\).

The computational results described in this paper show direct evidence of molecular rearrangements responsible for storage of about 50 % of the photon energy through isomerization of the retinyl chromophore, the preferential sense of rotation of the \(\phi\) (C11-C12) dihedral angle and the detailed chromophore-protein interactions responsible for the initial rotational torque and the storage mechanism. These results are particularly relevant to the development of a first principles understanding of the structure-function relationship of G-protein-coupled re-
ceptors at the detailed molecular level.

The paper is organized as follows. Section II A describes the preparation of the system. Secs. II B and II C outline the computational approach, including a description of the ONIOM hybrid methods and the optimization approach implemented for computing intermediate structures along the reaction pathway after electronic- and partial nuclear-relaxation. Section III presents QM/MM(MO:MM) results, organized in five subsections: Sec. III A addresses the structural integrity of the computational approach; Sec. III B reports results for the reaction energetics, including the computations of the energy-storage and its comparison to experimental values; Sec. III C discusses the results of molecular rearrangements; Sec. III D addresses the detailed chromophore-protein interactions responsible for the energy-storage mechanism; finally, Sec. III E reports the electronic excitations for rhodopsin and bathorhodopsin, as well as a comparison with the corresponding experimental values. Sec. IV summarizes and concludes.

II. METHODS

A. Structural Models

The computational models investigated in this paper are based on the refinement of the crystal structure of bovine rhodopsin (Protein Data Bank (PDB) accession code 1F88, monomer A), solved at 2.8 Å resolution. For the sake of comparison, we have also prepared model systems based on the more recent refinement of the 1F88 crystal structure (PDB accession code 1HZX, monomer A), also solved at 2.8 Å resolution. Model systems based on one or the other crystal structure typically have root mean squared (RMS) deviations of about 0.5 Å, taking into account both Cα atoms and side chains and show no significant difference in any of the results reported in this paper.

Starting from the PDB crystal structures, hydrogens are added using the molecular modeling program TINKER. The protonation of all titratable groups is standard. The rhodopsin cavity is set neutral, consistently with experiments. The Schiff-based linkage between Lys296 and the chromophore bears a net positive charge compensated by the Glu113 counterion (forming a salt-bridge). Amino acids Glu122, Asp83 and Glu181, within the protein core, are assumed to be neutral as indicated by FTIR experiments and UV-vis spectroscopic measurements of site-directed mutants. Although the PDB structures 1F88 does not contain a bound-water molecule near the chromophore binding site, we have also considered the presence of the bound-water molecule (Wat2b) consistently found by X-ray crystallographic studies of two monomers of bovine rhodopsin. The water molecule (Wat2b) fits in a cavity near the retinyl chromophore close to the salt-bridge between the Schiff-based NH(+) group and the Glu113 counterion. Finally, the two regular ends of the protein and the two artificial ends due to the missing amino acids from the X-ray structures in the third cytoplasmic loop (236-239) and in the C-terminal tail (328-333) are capped with NH₂ and CO₂⁻. Thus the model systems in the present calculations contain 5172 atoms.

B. ONIOM Hybrid Methods

The QM/MM(MO:MM) calculations reported in this paper are based on the ONIOM two-layer hydrogen link-atom scheme. The full-system of 5172 atoms is partitioned into two layers by placing a frontier at the Cα-Cα bond of the Lys296 side chain (i.e., two bonds beyond the C-NH(+) linkage). One layer corresponds to a reduced-system with 54 atoms, including 48 atoms of the retinyl chromophore, five atoms of Lys296 (NH, CH₂) and a link hydrogen atom that saturates the extra valence of the terminal -C-H₂. The other layer corresponds to the remainder of the protein. For the sake of comparison, we have also prepared model systems with the protein displaced by an additional bond (i.e., including the Cα-Cα bond of the Lys296 side chain into the reduced system) and we have observed no significant difference in any of the results.

The total energy $E$ of the system can be obtained according to the so-called ONIOM Molecular-Embedding (ONIOM-ME) approach from three independent calculations, as follows:

$$E = E_{\text{MM,full}} + E_{\text{QM,red}} - E_{\text{MM,red}}, \quad (2.1)$$

where $E_{\text{MM,full}}$ is the energy of the full-system computed at the molecular-mechanics level of theory, including the electrostatic interaction between all atomic charges; $E_{\text{QM,red}}$ is the energy of the reduced-system computed at the quantum-mechanics level of theory; and $E_{\text{MM,red}}$ is the energy of the reduced-system computed at the molecular-mechanics (MM) level of theory plus the electrostatic interaction with the remainder of the protein as described by the distribution of atomic charges implemented in the molecular-mechanics level of theory. It is important to note that the electrostatic interactions between the two layers are completely neglected in Eq. (2.1), since such interactions are included in both the $E_{\text{MM,full}}$ and $E_{\text{MM,red}}$ terms and therefore cancel.

The electrostatic interactions between the two layers are considered instead by implementing the so-called ONIOM Electronic-Embedding approach (ONIOM-EE) where such interactions are considered in both the $E_{\text{QM,red}}$ and $E_{\text{MM,red}}$ terms. The ONIOM-EE approach, therefore, introduces the electrostatic interaction between the two layers, including the polarization of the reduced-system by considering the perturbational effect of the surrounding charges in the quantum-mechanical calculation of the reduced-system.
The QM/MM calculations reported in this paper are carried out for the reduced-system embedded in the electric potential of atomic charges defined by the Amber force field\textsuperscript{66}, setting to zero the charges of atoms closer than four bonds from any atom in the reduced-system in order to avoid over-polarization of the reduced system. This approximation is subsequently validated in terms of the analysis of electrostatic chromophore-protein interactions (see Sec. III D). For the chromophore, ElectroStatic-Potential (ESP) atomic-charges are obtained at the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory by implementing a self-consistent approach, described in Sec. II C, for the optimized structures and electrostatic potentials obtained at that level of theory. The $S_0 \rightarrow S_1$ vertical electronic-excitation energies were computed at the time-dependent DFT ONIOM-EE (TD-B3LYP/6-31G*:Amber//B3LYP/6-31G*:Amber) level of theory\textsuperscript{67} (i.e., the ONIOM-EE (TD-B3LYP/6-31G*:Amber) level of theory after geometry optimization at the ONIOM-EE (B3LYP/6-31G*:Amber) level). Full geometry optimizations were carried out with the Gaussian03\textsuperscript{57} and Tinker programs\textsuperscript{60}, without boundary conditions or inclusion of solvent.

C. Reaction Path

In order to investigate the formation of bathorhodopsin in terms of intermediate structures we assume that the protein relaxation time is much longer that the reaction time (i.e., the time required for the retinyl structural rearrangements and relaxation to the ground electronic state). This assumption is consistent with both the experimental 200 fs reaction time\textsuperscript{26,27} and the observation that the degrees of freedom that are coupled to the rotation are mainly vibrational modes of the retinyl chromophore\textsuperscript{68}. In addition, we have observed that the energy-storage would be about 1/3 of the experimental value if the protein environment had enough time to fully relax and stabilize the intermediate structures along the reaction path as predicted by the ONIOM-EE (6-31G*:TD-B3LYP:Amber) level of theory.

The slower relaxation of the protein environment imposes steric constraints responsible for anchoring the \(\beta\)-ionone ring of the chromophore. Such constraints also establish a negative sense of rotation of the \(\phi(C11-C12)\) dihedral angle. Defining the rotation axis in the direction of the C11 \(-\) C12 vector, we observe that the Ala117 residue hinders the rotation towards positive angles (see Fig. 2), establishing the preferred negative sense of rotation followed upon photoisomerization. The presence of Ala117 is also responsible for an initial -10° twist of the \(\phi(C11-C12)\) dihedral angle, reported in Sec. II B. This picture is consistent with recent experimental studies of circular dichroism (CD)\textsuperscript{69,70}, \textit{ab initio} calculations of the CD spectrum\textsuperscript{71}, and pure molecular dynamics simulations\textsuperscript{22,43,72}.

Based on these observations, the reaction path is constructed along the direction of negative dihedral angles according to the following scheme:

- \textit{Amber-MM optimization:} Starting from the molecular structure of rhodopsin, prepared as described in Sec. II A and fully optimized as described in Sec. II B, the \(\phi(C11-C12)\) dihedral angle is changed by increments of 10° in the 0° to -180° total increment range. The 10° increments of the \(\phi(C11-C12)\) dihedral angle are defined by a 5° rigid-rotation of all atoms between the \(\beta\)-ionone ring and C10, including the methyl group at C9 and a -5° rigid-rotation of all atoms between C13 and C15, including the C13-methyl substituent group. After incrementing the \(\phi(C11-C12)\) dihedral angle according to such scheme, the total energy of the system is minimized subject to the constraint of fixed \(\phi(C11-C12)\) dihedral angle, allowing relaxation of all degrees of freedom of the retinyl chromophore, the Wat2b bound-water molecule and 20 atoms more beyond the Schiff-based linkage to Lys296. The rest of the protein structure is kept fixed. This optimization is implemented at the Amber-MM level, self-consistently with a refinement of ESP atomic charges for the retinyl chromophore. In order to describe the retinyl chromophore according to a realistic description of atomic charges, we have implemented a self-consistent approach at the ONIOM-EE level of theory. The ESP atomic charges computed for an optimized molecular structure are implemented in the next optimization cycle until con-
vergence. This approach typically converges within four iterations.

- **ONIOM-EE optimization**: The intermediate structures, obtained by optimization at the MM level of theory, are subsequently optimized at the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory using the same restrictions over relaxation of the protein implemented in the Amber-MM optimization.

The energy-storage is computed as the energy difference between the minimum energy structures obtained for the all-trans product and the 11-cis reactant sides at the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory. The electronic-excitation energy-shift is computed as the difference between the \( S_0 \rightarrow S_1 \) vertical excitations of rhodopsin and bathorhodopsin at the ONIOM-EE (TD-B3LYP/6-31G*:Amber //B3LYP/6-31G*:Amber) level of theory.

### III. RESULTS

#### A. Structural Integrity

In order to validate the structural integrity of the computational approach implemented in this paper, Fig. 3 quantifies the deviations between the rhodopsin molecular structure optimized at the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory and the X-ray crystallographic structure. The comparison includes the C-\( \alpha \) atoms located within a 20 A radius from each atom of the retinyl chromophore and the Schiff-based linkage to Lys296, a region that accounts for about 90% of the whole protein. Floppy side chains and residues beyond the 20 A radius were excluded to facilitate the comparison. Fig. 3 shows that the RMS \( \sim 0.75 \) A, with deviations larger than twice the RMS for only 5 atoms (all of them located at about 15 A from the chromophore). In addition, Fig. 3 shows that the residues with predominant interactions with the retinyl chromophore, including Glu113, Lys296 and Ala292 have even smaller deviations from their corresponding coordinates in the crystal X-ray structure (e.g., Glu113 (rms \( \sim 0.63 \) A), Lys296 (rms \( \sim 0.58 \) A) and Ala292 (rms \( \sim 0.50 \) A), and the chromophore (rms \( \sim 0.22 \) A)). Therefore, Fig. 3 indicates that the molecular structure obtained by geometry optimization at the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory is in very good agreement with the experimental X-ray crystal structure despite the negligence of the environment beyond the protein (including the solvent and the lipid bilayer) and the missing amino acid fragments in the third cytoplasmic loop (236-239) and in the C-terminal tail (328-333). The observed robustness of the molecular structure is probably due to the intrinsic stability of the seven transmembrane helical bundle that surrounds the covalently bound retinyl chromophore. The small deviations of the \textit{ab initio} coordinates relative to the crystallographic data also suggest that higher resolution structures should have very similar coordinates, at least for the C-\( \alpha \) backbone within 20 A from the retinyl chromophore.

#### B. Reaction Energetics

Fig. 4 shows the ground state energy profile of intermediate molecular structures along the 11-cis/all-trans isomerization reaction path, after electronic- and partial nuclear-relaxation as described in Sec. II C. Fig. 4 shows that the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory predicts a dihedral angle \( \phi(C11-C12)=-10^\circ \) for the optimized configuration of the 11-cis rhodopsin. This initial twist of the retinyl chromophore structure establishes a sense of rotation of the \( \phi(C11-C12) \) dihedral angle that is consistent with recent theoretical studies\textsuperscript{22,43}. In addition, Fig. 4 shows that the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory predicts an optimized configuration for the all-trans bathorhodopsin isomer with a dihedral angle \( \phi(C11-C12)=-165^\circ \) and 29 kcal/mol of storage-energy. The computed energy stored is in very good agreement with experimental values reported in the 32-35 kcal/mol energy range, with an estimated experimental error of about 3 kcal/mol (see Refs.\textsuperscript{13–15}). For the sake of comparison, we have also prepared model systems that do not include the bound-water molecule (Wat2b). The computed energy-storage in the absence of Wat2b is 30 kcal/mol, still in excellent agreement with experiments.

The favorable comparison with experiments suggests...
that the assumption of an isomerization time scale much shorter than the protein relaxation time is valid. In addition, the comparison indicates that the energy stored within 200 fs remains stored for much longer times (e.g., the measurement time), probably due to kinetic factors. Otherwise, complete relaxation of the protein environment would decrease the energy-storage to about 1/3 of the experimental value.

C. Molecular Rearrangements

Fig. 5 shows the underlying conformational changes due to the 11-cis/all-trans isomerization for three representative configurations, including the reactant 11-cis rhodopsin, an intermediate configuration along the path, and the product all-trans bathorhodopsin. Note that the isomerization reaction changes the $\phi$ (C11-C12) dihedral angle (highlighted in magenta) from -10° in the 11-cis isomer to -165° in the all-trans isomer, with a preferential negative sense of rotation determined by the steric Van der Waals interactions between Ala117 and the polyene chain at the C13 position. Note that the $\beta$-ionone ring, the Schiff-linkage (highlighted in blue) and the protein chain beyond the linkage (highlighted in grey) remain almost fixed throughout the isomerization, due to steric constraints in the rhodopsin cavity. These constraints induce a reorientation of the C13-methyl substituent group and torsion of the bonds at the Schiff-based linkage.

A quantitative analysis of the underlying rearrangements of dihedral angles along the chromophore polyene chain is presented in Table I, including the polyene torsions from C6 to C12 of the Lys296 side chain. Note that the comparative analysis of dihedral angles indicates that the product all-trans isomer is formed with a twisted and bent polyene chain, including out-of-the-plane distortions larger than 15° in the dihedral angles of the C7-C8, C9-C10, C12-C13, C14-C15 and C15-NH(+) chromophore bonds. Fig. 6 shows a superposition of the molecular structures of 11-cis rhodopsin $\phi$(C11-C12)=-10° intermediate structure at $\phi$(C11-C12)=-90° and all-trans bathorhodopsin $\phi$(C11-C12)=-165°, respectively.
TABLE I: Rearrangement of dihedral angles along the chromophore polyene chain, due to 11-\textit{cis} rhodopsin/all-\textit{trans} bathorhodopsin conversion, predicted by the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory.

<table>
<thead>
<tr>
<th>Dihedral angle</th>
<th>11-\textit{cis}</th>
<th>all-\textit{trans}</th>
<th>$\Delta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5-C6-C7-C8</td>
<td>-44</td>
<td>-37</td>
<td>8</td>
</tr>
<tr>
<td>C1-C6-C7-C8</td>
<td>135</td>
<td>139</td>
<td>4</td>
</tr>
<tr>
<td>C6-C7-C8-C9</td>
<td>-173</td>
<td>-157</td>
<td>16</td>
</tr>
<tr>
<td>C7-C8-C9-C(methyl 1)</td>
<td>-9</td>
<td>-13</td>
<td>4</td>
</tr>
<tr>
<td>C7-C8-C9-C10</td>
<td>167</td>
<td>162</td>
<td>6</td>
</tr>
<tr>
<td>C8-C9-C10-C11</td>
<td>179</td>
<td>-154</td>
<td>27</td>
</tr>
<tr>
<td>C9-C10-C11-C12</td>
<td>172</td>
<td>175</td>
<td>3</td>
</tr>
<tr>
<td>C10-C11-C12-C13</td>
<td>-10</td>
<td>-165</td>
<td>155</td>
</tr>
<tr>
<td>C11-C12-C13-C14</td>
<td>169</td>
<td>-175</td>
<td>17</td>
</tr>
<tr>
<td>C11-C12-C13-C(methyl 2)</td>
<td>-10</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>C12-C13-C14-C15</td>
<td>-179</td>
<td>-170</td>
<td>9</td>
</tr>
<tr>
<td>C13-C14-C15-N</td>
<td>165</td>
<td>-153</td>
<td>43</td>
</tr>
<tr>
<td>C14-C15-N-CE</td>
<td>170</td>
<td>-148</td>
<td>42</td>
</tr>
<tr>
<td>C15-N-CE-CD</td>
<td>100</td>
<td>109</td>
<td>9</td>
</tr>
</tbody>
</table>

FIG. 6: Superposition of the molecular structures of 11-\textit{cis} rhodopsin (brown) and all-\textit{trans} bathorhodopsin (white), optimized at the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory. The protein chain beyond the Schiff-linkage is represented by grey tubes. The Schiff-linkage is highlighted in blue and the C11-C12 bond in magenta.

induces a reorientation of the C13-methyl substituent group and torsion of the polyene chain at the protonated NH(+) Schiff-based linkaged to Lys296. Such torsion induces a reorientation of polarized bonds at the linkage, shown in Fig. 7, including the C15-H and N-H$^+$ bonds with partial positive charges on the H atoms. Note that the polarized bonds NH(+) and C15H are rotated in the all-\textit{trans} bathorhodopsin isomer, relative to their corresponding orientations in the reactant 11-\textit{cis} rhodopsin.

FIG. 7: Augmented view of the Schiff-linkage environment in terms of the superposition of rhodopsin (brown) and bathorhodopsin (white), surrounded by the distribution of residues with significant electrostatic contributions to the total energy-storage (see Fig. 8). The protein chain beyond the Schiff-linkage is represented by grey tubes and the Schiff-linkage is highlighted in blue. The red-dashed arrows indicate the reorientation of polarized bonds responsible for the displacement of the net positive charge at the linkage, including the N-H$^+$ and C15-H bonds (only the hydrogen atoms of these two bonds are represented by spheres). The negative charge density is highlighted in blue.

The analysis of ESP atomic charges indicates that these two polarized bonds account for most of the net positive charge at the linkage. Therefore, due to the reorientation of the bonds, the net positive charge of the chro-
mohore is farther away from the Glu113 counterion in bathorhodopsin than in rhodopsin, even when the linkage itself remains almost fixed due to the cavity’s steric constraints.

The molecular rearrangements described in Figs. 5—7 and Table I indicate that the isomerization reaction produces a highly distorted all-trans retinyl chromophore (e.g., significantly bent and twisted relative to the linear molecular structure of the all-trans retinal isomer in the gas-phase). Therefore, although the seven transmembrane helical bundle isolates the retinyl chromophore from interactions with the solvent, the 11-cis/all-trans isomerization reaction does not proceed in the usual sense as expected from the reaction in the gas phase. In the protein, significant distortions are necessary to fit the bathorhodopsin isomer in the rhodopsin binding pocket.

The underlying molecular rearrangements predicted by the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory can be compared to the molecular rearrangements in the “bicycle-pedal” pathway proposed and refined by Warshel many years ago, where the 180° rotation around the C11-C12 bond is compensated by distortions larger than 15°, 42°, and 43° twists around the C9-C10 and C15-N bonds. As described earlier in this section, the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory predicts that the isomerization reaction involves a 155° rotation around the C11-C12 bond, compensated by distortions larger than 15° in the dihedral angles along the C7-C8, C9-C10, C12-C13, C14-C15 and C15-NH(+) bonds. Remarkably, however, the larger distortions predicted by the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory correspond to 27°, 42°, and 43° twists around the C9-C10, C14-C15 and C15-N bonds, respectively, in partial agreement with the reaction in the earlier model. The torsion around the C15-N bond, however, is crucial for the reorientation of polarized bonds and is induced by the steric constraints introduced by the turn of the protein backbone at the Cα atom of Lys296. Otherwise, torsion of the dihedral angle along the N-Cα bond of Lys296 could be more favorable energetically.

D. Chromophore-Protein Interactions

The molecular rearrangements described in Sec. III C indicate that a significant storage of strain energy must be observed due to the formation of bathorhodopsin in a highly distorted all-trans conformation. In addition, Sec. III C indicates that the isomerization must store electrostatic energy due to the separation of the positive charge in the Schiff-base from the negatively charged Glu113 counterion. It is important to mention here that the ESP atomic charges in the protonated Schiff-based retinyl chromophore are almost identical for 11-cis rhodopsin and all-trans bathorhodopsin, as predicted by the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory. Therefore, electrostatic contributions to the total energy-storage due to changes in atomic charges within the polyene chain are negligible. In contrast, the reorientation of the polarized bonds C15-H and N-H+ accounts for a significant electrostatic contribution to the total energy-storage, due to the displacement of the net positive charge relative to the Glu113 counterion. The charge separation process is based on rotation of the polarized bonds C15-H and N-H+, with only a minor displacement of the C15-N linkage relative to the Glu113 counterion. Therefore, the underlying mechanism is significantly different from charge separation mechanisms based on displacement of the polyene chain linkage away from the carbonylate counterion to a nonpolar environment.

The energy-storage computed at the ONIOM-ME (B3LYP/6-31G*:Amber) level of theory is 11 kcal/mol. As discussed in Sec. II B, however, the ONIOM-ME level of theory neglects the electrostatic interaction between the reduced-system and the remainder of the protein. Therefore, the difference between the storage-energy computed at the ONIOM-EE and ONIOM-ME levels of theory provides an estimate of the electrostatic contribution to the total storage-energy. Since the storage computed at the ONIOM-EE level of theory is 29 kcal/mol, it is estimated that about 62 % of the total energy-storage corresponds to electrostatic contributions.

In order to elucidate the electrostatic influence of individual residues in the protein environment, we have computed the electrostatic contributions of individual residues to the total energy-storage. The electrostatic interactions between the chromophore and each residue was obtained in terms of the distribution of ESP atomic charges in the retinyl chromophore obtained at the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory. Fig. 8 shows the electrostatic contribution of each individual residue to the total energy-storage.
hydrogen-bonded water molecule, including the Glu113 counterion, Ser186, Cys187, Ala292 and Wat2b. The results also indicate that indeed the largest electrostatic contribution to the energy-storage results from the separation of positive charge from the Glu113 counterion. In addition, Fig. 8 shows that the electrostatic interactions due to Ser186 and Wat2b stabilize the product bathorhodopsin relative to rhodopsin and, therefore, reduce the total energy-storage. In contrast, the electrostatic interactions due to Glu113, Cys187 and Ala292 are responsible for increasing the total energy-storage. Fig. 7 shows the distribution of these important residues in the rhodopsin binding pocket, where the negative charge density is highlighted in blue.

E. Electronic Excitations

Table II reports $S_0 \rightarrow S_1$ vertical electronic-excitation energies, computed at the ONIOM-EE (TD-B3LYP/6-31G*:Amber) level of theory, for the molecular structures of 11-cis rhodopsin and all-trans bathorhodopsin optimized at the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory. In addition, Table II compares the computed vertical excitations to the corresponding experimental values. Table II also compares the $S_0 \rightarrow S_1$ electronic-excitation of 11-cis rhodopsin to recently reported QM/MM (CASPT2/CASSCF/6-31G*:Amber) calculations by Olivucci and co-workers. It is shown that the ONIOM-EE (TD-B3LYP//B3LYP/6-31G*:Amber) level of theory predicts the $S_0 \rightarrow S_1$ electronic-excitation energy-shift, due to the 11-cis/all-trans isomerization, in excellent agreement with experiments. However, the individual electronic excitations of rhodopsin and bathorhodopsin are overestimated by about 10% relative to the corresponding experimental values. Such overestimation is, however, common to the QM/MM (CASPT2/CASSCF/6-31G*:Amber) level, and has also been observed in recent calculations of vertical-excitations in bacteriorhodopsin, reported by Vreven and Morokuma. It is important to note that while all of these QM/MM studies have considered the polarization of the reduced-system, the self-consistent polarization of the protein has been neglected. Therefore, such an approximation can be responsible for the observed overestimation of vertical excitation energies. Further progress in the description of electronic excitation requires consideration of the self-consistent protein polarization, e.g. by expanding the reduced system with the residues with predominant electrostatic interactions with the retinyl chromophore.

IV. CONCLUSIONS

We have implemented ONIOM (MO:MM) hybrid methods in conjunction with high-resolution structural data of bovine rhodopsin to investigate the primary photochemical event in vision. We have assumed that such process involves 11-cis/all-trans isomerization of the retinyl chromophore in rhodopsin within a time scale much shorter than the time required by the protein environment to relax and fully stabilize the intermediate structures along the reaction path.

We have shown that the analysis of intermediate structures along the 11-cis/all-trans isomerization path, after electronic- and partial nuclear-relaxation, reveals the detailed molecular rearrangements of the retinyl chromophore in the rhodopsin binding pocket environment; the preferential rotational direction of the $\phi$(C11-C12) dihedral angle; and the detailed chromophore-protein interactions responsible for both the initial rotational torque and the energy-storage mechanism. We have predicted that the $\phi$(C11-C12) dihedral angle ranges from -10° in the 11-cis isomer to -165° in the all-trans isomer. We have shown that the preferential sense of rotation of the $\phi$(C11-C12) dihedral angle is along the direction of increasingly negative angles, while the positive rotation is hindered by the steric Van der Waals interactions between Ala117 and the polyene chain at the C13 position.

We have shown that the all-trans isomer is formed with a twisted and bent polyene chain, due to spatial constraints in the rhodopsin binding pocket, including out-of-the-plane distortions larger than 15° in the dihedral angles of the C7-C8, C9-C10, C12-C13, C14-C15 and C15-NH(+) chromophore bonds. The spatial constraints include the steric interactions of the three methyl-substituent groups of the $\beta$-ionone ring with the surrounding residues, the steric interaction of Ala117 with the polyene chain at the C13 position, and the steric constraints beyond the linkage to Lys296 due to the almost perpendicular orientation of the protein chain with respect to the C11-C12 rotational axis in the retinyl chromophore.

We have shown that the energy-storage predicted by the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory is 29 kcal/mol (i.e., approximately 50% of the photoexcitation energy), in excellent agreement with experimental measurements reported in Refs. (32–35 ± 3 kcal/mol). This comparison suggests that the assumption of an isomerization time scale much shorter than the protein relaxation time is valid. In addition, we conclude that the energy stored within 200 fs remains stored for much longer times (e.g., the measurement time), probably due to kinetic factors. Otherwise, complete relaxation of the protein environment would decrease the energy-storage to about 1/3 of the experimental value.

We have shown that the structural distortions of the all-trans bathorhodopsin isomer account for 40% of the energy stored during the primary event, while the remaining 60% is electrostatic energy due to stretching of the salt-bridge between the protonated Schiff-base and the Glu113 counterion. We have indicated that the salt-bridge stretching mechanism involves reorientation of the positively charged groups due to torsion of the polyene chain at the linkage to Lys296, rather than involving
TABLE II: Electronic-excitation energies, in kcal/mol, for S$_0$ $\rightarrow$ S$_1$ transitions in rhodopsin and bathorhodopsin.

<table>
<thead>
<tr>
<th>Method</th>
<th>Excitation Energies, $\Delta E$</th>
<th>$\Delta\Delta E = \Delta E_{\text{rhod.}} - \Delta E_{\text{batho.}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONIOM-EE (TD-B3LYP//B3LYP/6-31G*:Amber)</td>
<td>$\Delta E_{\text{rhod.}}$</td>
<td>$\Delta E_{\text{batho.}}$</td>
</tr>
<tr>
<td></td>
<td>63.5</td>
<td>60.3</td>
</tr>
<tr>
<td>QM/MM (CASPT2//CASSCF/6-31G*:Amber)</td>
<td>64.1</td>
<td>54.0</td>
</tr>
<tr>
<td>Experimental values$^a$</td>
<td>57.4</td>
<td>54.0</td>
</tr>
</tbody>
</table>

$^a$ reference$^{73}$  $^b$ reference$^{18}$

any significant displacement of the polyene chain at the Schiff-based linkage relative to the Glu113 counterion, or redistribution of charges within the chromophore. In addition, we have shown that a hydrogen-bonded water molecule (Wat2b), consistently found by X-ray crystallographic studies, can assist the salt-bridge stretching process by stabilizing the reorientation of positively charged groups. It is important to note, however, that in the absence of the Wat2b molecule the underlying molecular rearrangements are still very similar and the energy-storage predicted by the ONIOM-EE (B3LYP/6-31G*:Amber) level of theory (30 kcal/mol) is still in excellent agreement with experiments.

Furthermore, we have observed that the direct correlation between molecular rearrangements and the electrostatic contribution to the total energy-storage is due to the fact that the atomic charges in the protonated Schiff-based retinyl chromophore are almost identical for rhodopsin and bathorhodopsin. Therefore, the electrostatic contribution to the total energy storage due to a redistribution of atomic charges within the polyene chain is negligible.

We have shown that the ONIOM-EE (TD-B3LYP/6-31G*/B3LYP/6-31G*:Amber) level of theory overestimates the electronic S$_0$ $\rightarrow$ S$_1$ vertical excitation energies for both the reactant 11-cis rhodopsin and the isomerization product all-trans bathorhodopsin by about 10%, relative to the corresponding experimental values. This description is, nonetheless, in excellent agreement with the QM/MM (CASPT2//CASSCF/6-31G*:Amber) level of theory and still predicts an electronic-excitation energy-shift $\Delta\Delta E = 3.2$ kcal/mol in excellent agreement with experimental measurements (3.4 kcal/mol). We have concluded that further progress in the description of the system requires addressing the influence of the self-consistent polarization of the protein. Work in progress in our group involves the development of a practical approach for including the self-consistent polarization of the protein at the ONIOM-EE level of theory.

We have demonstrated that the predominant electrostatic contributions to the total energy-storage result from the interaction of the protonated Schiff-based retinyl chromophore with four surrounding residues and a hydrogen-bonded water molecule, including the Glu113 counterion, Ser186, Cys187, Ala292 and Wat2b. These findings suggest that selective artificial mutations of any of these four amino acids could have significant implications on the efficiency of the underlying photo-transduction mechanism. Furthermore, we conclude that most of the protein polarization effect on the primary photochemical event could also be modeled by expanding the reduced 54-atom system with Glu113, Ser186, Cys187, Ala292, Ala117 and Wat2b. Studies of expanded reduced systems would also allow one to investigate the ONIOM inter-layer boundary conditions as a function of the size of the reduced system.

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