Resonance Raman Analysis of the Mechanism of Energy Storage and Chromophore Distortion in the Primary Visual Photoproduct†

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ABSTRACT: The vibrational structure of the chromophore in the primary photoproduct of vision, bathorhodopsin, is examined to determine the cause of the anomalously decoupled and intense C11=C12 hydrogen-out-of-plane (HOOP) wagging modes and their relation to energy storage in the primary photoproduct. Low-temperature (77 K) resonance Raman spectra of Glu181 and Ser186 mutants of bovine rhodopsin reveal only mild mutagenic perturbations of the photoproduct spectrum suggesting that dipolar, electrostatic, or steric interactions with these residues do not cause the HOOP mode frequencies and intensities. Density functional theory calculations are performed to investigate the effect of geometric distortion on the HOOP coupling. The decoupled HOOP modes can be simulated by imposing ~40° twists in the same direction about the C11=C12 and C12=C13 bonds. Sequence comparison and examination of the binding site suggests that these distortions are caused by three constraints consisting of an electrostatic anchor between the protonated Schiff base and the Glu113 counterion, as well as steric interactions of the 9- and 13-methyl groups with surrounding residues. This distortion stores light energy that is used to drive the subsequent protein conformational changes that activate rhodopsin.

Photoactive proteins play a vital role in a variety of light-energy and light-signaling processes that are characterized by picosecond to femtosecond light-driven structural changes of a protein-bound chromophore followed by activated conformational changes of the protein. In systems such as rhodopsin (Rho)1 (1), bacteriorhodopsin (2, 3), halorhodopsin (4), photoactive yellow protein (5), and phytochrome (6, 7), primary high-energy intermediates are produced with structurally perturbed chromophores. What is the nature of the protein—chromophore interactions that store the energy needed to drive subsequent protein conformational changes? To address this question, we have studied the photoactivation of rhodopsin, a dim-light photoreceptor, using a multidisciplinary approach that integrates Raman spectroscopy, mutagenesis, density functional theory (DFT) calculations, and bioinformatic analysis.

Rho is a paradigmatic G protein-coupled receptor, whose 7-transmembrane helical structure forms a binding pocket for 11-cis-retinal, which is covalently linked to Lys296 on helix 7. Within 200 fs after photoexcitation (8, 9), the 11-cis-retinal protonated Schiff base (PSB) isomerizes to all-trans-retinal (ATR) with a quantum yield of 0.65 to form the primary photoproduct, Bathorhodopsin (10, 11), which stores ~30 kcal/mol or ~60% of the incident photon energy (Figure 1b) (12–14). Subsequently, the highly distorted chromophore relaxes, and the transduced energy is used to drive conformational changes in the opsin protein (15–18).

Recently, we found that this structural evolution includes a retinal counterion switch from Glu113 in the dark state to Glu181 in the metarhodopsin I (Meta I) state (19).The Schiff base is then deprotonated upon Meta II formation, and Meta II couples with transducin to trigger the visual signal cascade (20, 21).

Structural investigations on the primary photoproduct of rhodopsin have strived to determine how the photoisomerization of the 11-cis-retinal PSB chromophore is catalyzed and how the light energy is used to drive subsequent protein conformational changes. In 1974, Oseroff and Callender obtained the first Raman spectrum of Batho (22), revealing unique vibrational lines at ~850, 875, and 920 cm−1 that are markers for the highly energetic and structurally distorted chromophore (Figure 1b). Proton transfer at the Schiff base and the formation of a tautomerized chromophore in the primary photoexcitation step were among the structures first proposed to explain the unique properties of Batho (23, 24). Subsequent Raman experiments revealed a normal protonated...
Schiff base structure in Batho that disproved these ideas (25). Further measurements, exploiting isotopically labeled retinal derivatives from the Lugtenburg group, completed the assignment of the unique Batho lines to hydrogen-out-of-plane (HOOP) wagging modes of the chromophore (26-28). Hydrogen-out-of-plane (HOOP) wags across a double bond generally couple strongly producing an in-phase mode at ~970 cm\(^{-1}\) and a lower wavenumber out-of-phase combination. However, the C\(_{11}\)=C\(_{12}\) HOOP modes of the Batho chromophore are unexpectedly decoupled producing a nearly isolated C\(_{11}\)H wag at 920 cm\(^{-1}\) and C\(_{12}\)H wag at 858 cm\(^{-1}\). Since this assignment was achieved, the origin of this key structural observation about the structure of the Batho chromophore has remained unresolved despite a wide variety of theoretical models and experimental studies (1, 13, 29-31).

The recent crystal structure of bovine rhodopsin (32) prompted us to reexamine the origin of this HOOP mode perturbation. A surprising feature of the structure is that the \(\beta4\) sheet of extracellular loop 2 (E2) aligns with the ethylenic chain of 11-cis-retinal, placing a potentially charged residue (Glu181) 4.7 Å away from C\(_{12}\) and may interact significantly with the chromophore. The strong electrostatic interaction of the PSB with its Glu113 counterion, as well as the hydrogen-bond chain connecting Glu113, Glu181, Ser186, and the retinylidene PSB via two water molecules, is also indicated.

Here, we have obtained low-temperature (77 K) resonance Raman spectra of Glu181 mutants of bovine rhodopsin to test the hypothesis that an electrostatic or a dipolar interaction between Glu181 and chromophore decouples the C\(_{11}\)=C\(_{12}\) HOOP modes in Batho. Surprisingly, the spectra of the Glu181 mutants exhibit only mild mutagenic alterations of the frequencies of the decoupled C\(_{11}\)=C\(_{12}\) HOOP modes in the Batho chromophore, indicating that direct electrostatic, dipolar, or steric interaction with Glu181 is not significant. Having eliminated this possibility, we then perform DFT calculations of the photoproduct vibrational structure to explore whether the previously identified (27) geometric distortion might be sufficient to explain the decoupled HOOP frequencies. A unique set of twists is identified that reproduces the characteristic decoupled vibrational frequencies of Batho. Furthermore, a comparison of Batho Raman spectra of a variety of native visual pigments and of bovine rhodopsin analogues viewed in the context of their protein sequences allows us to propose that steric interactions with the 9- and 13-methyl groups, as well as the electrostatic anchoring of the protonated Schiff base group are the origin of the structural constraints that cause the HOOP decoupling. Protein-mediated distortions of conjugated C\(=\)C bonds are thus implicated as a general mechanism for storing the light
energy that is used to drive subsequent conformational changes in light-activated proteins.

EXPERIMENTAL SECTION

Site-directed point mutations were prepared using the QuickChange method (Stratagene) and verified by sequencing. The mutant pigments were transiently expressed in COS cells, harvested, regenerated with 11-cis-retinal, and purified by an immunoaffinity adsorption procedure (37, 38). Pigments were prepared in 100 mM NaCl and 0.1% dodecyl maltoside (DM) in 50 mM phosphate buffer at pH 6.8 and concentrated to ~1.5 OD at 500 nm. The pigment solutions were mixed with an equal volume of glycerol and then transferred to a 600 μm ID capillary that was cooled to ~77 K in a thermally insulated Harney–Miller cell by chilled nitrogen gas.

Resonance Raman spectra of pigments in the Batho state were obtained using a 496- or 488-nm probe beam from an argon ion laser (Spectra-Physics 2020) and a coaxial 568-nm pump beam from krypton ion laser (Spectra-Physics 2025). The pigment solutions were irradiated by a blue probe beam to obtain Raman spectra of a steady-state mixture of approximately 25% Rho, 15% isorhodopsin (Iso), and 60% Batho (22). The coaxial 568-nm pump beam was added at sufficient power to drive down the Batho concentration, facilitating the assignment of the Batho spectra (39). The scattering was dispersed by a double spectrograph (Spex 1400) and detected by a CCD (LN/CCD-1100/PB, Princeton Instruments). The spectra were calibrated against cyclohexane and cyclohexanone. The reported frequencies are accurate to 2 cm⁻¹, and the spectral resolution is 2 cm⁻¹.

DFT calculations were carried out using Gaussian 98 (40) with implementation of Becke’s Three Parameter Hybrid Functional using the Lee, Yang, and Parr Correlation Functional (B3LYP) (41). This level of theory with the 6-31G(d) basis set has been used (42–45) to calculate normal-mode frequencies and Raman activities of 30-60 atom organic molecules. The initial geometry for 6-s-cis ATR was taken from the crystal structure (46). Dihedral angles about the C₁₁=C₁₂ and/or C₁₃=C₁₂ bonds were set as constants, while the remaining degrees of freedom were allowed to relax in the electronic structure optimization (47). These optimizations show that twists about the C₁₁=C₁₂ and C₁₂=C₁₃ bonds trigger deviations of the neighboring ethylenic chain from planarity. For example, setting the dihedral twists of the C₁₁=C₁₂ and C₁₂=C₁₃ bonds to ~40° in the ATR–PSB model induces twists about the C₁₃=C₁₀ (~6.0°), C₁₀=C₁₁ (8.8°), C₁₃=C₁₄ (~1.9°), and C₁₄=C₁₅ (~1.9°) bonds upon structural optimization. The normal-mode frequencies were then calculated with the optimized constrained geometry using the same level of theory and basis set. A linear scaling factor of 0.9538 was determined by a least-squares fit of the 800–1050 cm⁻¹ region of the calculated HOOP frequencies to the experimentally observed frequencies with the y intercept equal to 0 (48). The torsional constraints on the C₁₁=C₁₂ and C₁₂=C₁₃ bonds in the calculation project primarily onto displacements along the low-frequency (~550 cm⁻¹) dihedral torsions (49) that are energetically well-separated from the HOOP region (830–970 cm⁻¹); their effect on the HOOP modes is thus negligible.

The masses of the coupled hydrogens other than C₁₁H and C₁₂H were set to 10.0 amu to drop their corresponding wagging frequencies by ~300 cm⁻¹, thereby removing the interaction of the heavy HOOP wagging and rocking modes with the individual C₁₁ and C₁₂ HOOP modes. The resulting frequencies allow us to study the intrinsic effect of dihedral twists at C₁₁=C₁₂ and C₁₂=C₁₃ on the C₁₁=C₁₂ HOOP, decoupling without interference from mixing and/or coupling with other vibrational modes (28). A mass of >2.0 was chosen because we found that the deuterated rocking modes dropped to the 900–960 cm⁻¹ (51) region and complicated the interpretation of the calculated HOOP results. Once the intrinsic effect of torsional distortion on the HOOP modes was identified, key limiting calculations were repeated with normal hydrogen to verify the validity of our conclusions.

The calculations were repeated for the ATR–PSB to determine whether the twisting results are also pertinent for the PSB chromophore. The initial ATR–PSB structure was constructed by replacing the terminal oxygen of ATR with a NHCH₂CH₂ group using the anti C=N configuration. To mimic the electronic properties of the Glu113 counterion, a Cl atom was added initially in line with the N–H bond and allowed to minimize. The ion pair was maintained throughout the calculation, as determined by a Mulliken atomic charge analysis. The calculated normal modes were visualized in Molden (50).

RESULTS

The resonance Raman spectra of Rho and selected mutants (E181Q and S186A) at ~77 K are presented in Figure 3. In Figure 3a, irradiation of wild-type Rho with the 496-nm probe beam creates a steady-state mixture of Rho, Iso, and Batho. Two intense ethylenic bands at 1537 cm⁻¹ for Batho and at 1550 cm⁻¹ for the mixture of Rho and Iso are observed. The characteristic decoupled HOOP vibrational bands of Batho at 857, 875, and 920 cm⁻¹ are also prominent. A coaxial 568-nm pump beam was then added to drive down the Batho concentration. The intensity of the Batho ethylenic band (1537 cm⁻¹) and the Batho HOOP bands (856, 874, and 920 cm⁻¹) decreases, confirming a depletion of Batho in the steady-state mixture. Subtracting the pump + probe spectrum from the probe-only spectrum to eliminate the 1550 cm⁻¹ band yields the Batho spectra. When this experiment is performed on the E181Q mutant with a 496-nm probe beam and the Ser186 mutant with a 488-nm probe beam, the two ethylenic bands are not as distinct. However, the intense decoupled HOOP bands in the 850–950 cm⁻¹ region are clearly observed. Addition of the 568-nm pump beam drives down the concentration of the Batho photoproduct allowing subtractions to yield the Batho spectra of the mutants. The observation of the steady-state mixture and of the characteristic HOOP modes of the primary photoproducts for the mutant pigments at low temperature (77 K) demonstrates that their primary photochemistry is very similar to that of the native pigment.

Figure 4 presents expanded views of the resonance Raman spectra of wild-type (WT) Batho and its E181D, -Q, and -F mutants. The Batho spectrum of the E181D mutant exhibits the same ethylenic stretching, C=N stretching, and HOOP wagging vibrational frequencies as the WT (±2 cm⁻¹), suggesting that the E181D mutagenic perturbation to the structure of the primary photoproduct is negligible. The Batho spectra of E181Q and E181F are more significantly
altered. The C=N stretching shifts from 1657 to 1664 cm\(^{-1}\) for E181Q and to 1663 cm\(^{-1}\) for E181F, suggesting that the PSB group is in a stronger hydrogen-bonded environment in the mutant pigments (52, 53). The C=C stretching frequency is shifted from 1536 to 1531 cm\(^{-1}\) for both E181Q and E181F, suggesting a red shift in their UV–vis absorption spectra. The mild perturbations of the mutant Batho spectra are consistent with a protonated (neutral) Glu181 in the Batho state in agreement with our previous conclusion that Glu181 is protonated in the dark state (19, 36). Most importantly, all Glu181 mutant Batho spectra exhibit the characteristic 3 bands in the HOOP region (850–950 cm\(^{-1}\)) within 4 cm\(^{-1}\) of their native frequencies. The consistent frequency and intensity patterns of the HOOP modes suggest that the C\(_{11}\)-HOOP wags in the mutants remain decoupled despite these mutations.

Figure 3 presents a spectral decomposition of the HOOP region of the Batho spectra of the Glu181 and Ser186 mutants compared with the WT pigment. The E181D and E181F spectra exhibit the same C\(_{11}\)-HOOP frequency (920 cm\(^{-1}\)) as the WT within the experimental error (2 cm\(^{-1}\)), while the S186A and S186I spectra exhibit an upshift of 4 cm\(^{-1}\). The bandwidths of the 920-cm\(^{-1}\) line for the mutants (20–23 cm\(^{-1}\)) are broader than that for the WT (13 cm\(^{-1}\)). We reproducibly found that two Gaussian peaks at 916 and 932 cm\(^{-1}\) are necessary to fit the C\(_{11}\)-HOOP band of E181Q. This suggests that there might be two different Batho chromophore structures for E181Q, though we cannot exclude the possibility that an incomplete subtraction of the Rho and Iso contribution leads to the observation of the 932-cm\(^{-1}\) line (see Figure 3). The C\(_{10}\)-HOOP vibrational frequency of the mutants is the same as that of the WT within the experimental error (2 cm\(^{-1}\)), showing that these mutations have essentially no effect on the chromophore structure near C\(_{10}\). The third observable HOOP band at ~856 cm\(^{-1}\) is decomposed to two Gaussian peaks corresponding to the C\(_{14}\)-HOOP at ~850 cm\(^{-1}\) and C\(_{12}\)-HOOP at ~858 cm\(^{-1}\). The decomposed C\(_{12}\)- and C\(_{14}\)-HOOP bands of the mutants are
within 4 cm\(^{-1}\) of the WT control. Lower wavenumber lines at \(\sim 820\) and \(\sim 835\) cm\(^{-1}\), previously assigned as combinations of out-of-plane modes (28), are more pronounced in the E181Q, E181F, and S186A spectra. The intensity ratios of the \(\sim 856-875\) cm\(^{-1}\) bands in these pigments are also similar, indicating a geometric perturbation to the ethylenic C\(_{11}\)-C\(_{12}\) HOOP modes.

**Vibrational Modeling.** Because Glu181 and Ser186 mutations did not produce a significant alteration of the Batho HOOP modes and, on the basis of the crystal structure, there are no other obvious perturbing residues near the C\(_{11}\)=C\(_{12}\) bond (see Figure 2), we used DFT calculations to explore whether conformational and/or configurational distortion might be sufficient to produce the unique vibrational frequencies of the C\(_{11}\)- and C\(_{12}\)-HOOP modes in Batho. In the calculations, the dihedral twists about the C\(_{11}\)=C\(_{12}\) and/or C\(_{12}\)=C\(_{13}\) bonds were fixed and then the chromophore structure was optimized. Figure 6 presents a selection of the calculated intrinsic C\(_{11}\)-H and C\(_{12}\)-H wagging frequencies when various fixed twists are applied. We were looking for a perturbation that would produce a significant reduction of the intrinsic C\(_{12}\)-H wagging frequency thereby producing a large splitting between the intrinsic C\(_{11}\)-H and C\(_{12}\)-H wagging modes. For the ATR calculation, simultaneous opposite sense twists as large as 40° (e.g., 40°, −40°) give insignificant lowering of the C\(_{12}\)-H wag compared with the experimental splitting of \(\sim 60\) cm\(^{-1}\). Twisting at just the C\(_{11}\)=C\(_{12}\) bond by up to 20° also induces an insignificant frequency lowering in the ATR calculations. The intrinsic C\(_{12}\)-H wag frequency is more sensitive to twists about the C\(_{12}\)=C\(_{13}\) bond; a 20° dihedral twist lowers this mode by 13 cm\(^{-1}\) in ATR. Twisting in the same sense about both the C\(_{11}\)=C\(_{12}\) and C\(_{12}\)=C\(_{13}\) dihedral angles consistently dropped the C\(_{12}\)-H wag by \(\sim 1.5\) cm\(^{-1}\) per degree with a \(< 10\) cm\(^{-1}\) shift of the C\(_{11}\)-H wag. The −40°/−40° calculation gives a C\(_{12}\)-H=C\(_{12}\)-H splitting of 63 cm\(^{-1}\), yielding the closest match to the Batho experiment of the 15 calculated structures.

Similar results were obtained for the ATR−PSB calculations, which are presented in the shaded region of Figure 6. Twists about C\(_{11}\)=C\(_{12}\) and C\(_{12}\)=C\(_{13}\) in the opposite sense (40°, −40°) had no significant effect, and twisting just one bond by up to 40° produced a 20 cm\(^{-1}\) frequency reduction only when the C\(_{12}\)=C\(_{13}\) bond was distorted. The C\(_{11}\)-H and C\(_{12}\)-H wagging modes were more effectively decoupled by applying twists about both bonds simultaneously, and the −40°/−40° structure gives a 61 cm\(^{-1}\) splitting, which closely matches the experimental value of 63 cm\(^{-1}\) for Batho. The effect of dihedral twists at other positions was also explored extensively but none of them significantly alter the C\(_{11}\)=C\(_{12}\) HOOP modes.

Figure 7 presents the relative contributions of the C\(_{11}\)-H and C\(_{12}\)-H wags to different normal modes for the ATR−PSB chromophore using conventional hydrogen masses. For the planar (0, 0) molecule, the C\(_{11}\)- and C\(_{12}\)-HOOP wags are strongly and roughly equally coupled producing two in-phase modes at 960–970 cm\(^{-1}\). Additional out-of-phase combinations are found from 825 to 850 cm\(^{-1}\) that are more dominated by C\(_{12}\)-H character consistent with the experiment (28, 48). For the highly twisted (−40, −40) molecule, the pattern collapses to an isolated C\(_{11}\)-H wag at \(\sim 930\) cm\(^{-1}\).
DISCUSSION

To identify the cause of the decoupling of the $C_{11}$-$C_{12}$ HOOP modes in the primary photoproduction of vision, we first hypothesized that Glu181 and Ser186, being dipolar residues that are near to the center of the chromophore, might perturb the photoproduction and cause the characteristic anomalous HOOP frequencies. The Batho Raman spectra of Glu181 and Ser186 mutants clearly demonstrate only mild mutagenic perturbation to the vibrational structure, indicating that direct dielectric, charge, or steric interaction with the polar residues Glu181 or Ser186 does not cause the decoupling of the $C_{11}$-$C_{12}$ HOOP modes.

We then explored whether the decoupling of the $C_{11}$-$C_{12}$ HOOP modes could be achieved by pure geometric distortion. Although NMR studies by Smith’s group (29, 30) as well as theoretical studies (13, 31) have suggested that geometric distortion of the chromophore is important in the energy-storage mechanism, no detailed geometry and specific chromophore–protein interactions have been put forward to account for the HOOP frequencies. The highly unique and unusual vibrational structure of Batho has thus remained an enigma since its first observation in 1974 (22). To address this challenge, we calculated the Raman vibrational frequencies of the $C_{11}$-$C_{12}$ and $C_{12}$-$C_{13}$ HOOP modes with a wide variety of distortions from planarity. The experimentally observed $C_{11}$-$C_{12}$ decoupling is successfully simulated only when dihedral twists of $\sim 40^\circ$ were imposed about the $C_{11}$-$C_{12}$ and $C_{12}$-$C_{13}$ bonds in the same sense for both ATR and ATR–PSB. Our calculations further indicate that the terminal group does not mechanistically change the nature of the distortions that cause the $C_{11}$-$C_{12}$ decoupling. The calculated ATR and ATR–PSB structures have a torsional energy of 14.1 and 15.6 kcal/mol, respectively, compared to a total of $\sim 30$ kcal/mol in Batho (12, 14). This calculated energy is only a qualitative estimate because all of the protein interactions have not been included, but this result does indicate that torsional distortions are an important energy-storage component. The absolute sense of the twisting cannot be determined by our calculations alone because the $(+, +)$ and $(-, -)$ twists are equivalent in the calculation. The twists about the $C_{11}$-$C_{12}$ and $C_{12}$-$C_{13}$ bonds in Batho propagate along the ethylenic chain causing the neighboring bonds to be distorted from planarity as well. The resulting out-of-plane displacement at the $C_{9}$-$C_{10}$-$C_{10}$-$C_{11}$, $C_{12}$-$C_{13}$-$C_{14}$, and $C_{14}$-$C_{15}$ bonds is anticipated to be the origin of the HOOP intensities at $C_{10}$-$H$ and $C_{14}$-$H$ (28).

**Protein-Mediated Distortion.** Because distortion along the ethylenic chain is sufficient to cause the $C_{11}$-$C_{12}$ HOOP decoupling in the Batho chromophore, it is pertinent to examine the crystal structure to consider the molecular origin of this distortion. A comparison of the Batho Raman spectra and the protein sequences of a variety of visual pigments provides insight into the origin of the distortion. Figure 8 compares the positions of the HOOP bands in the Batho spectra of a variety of visual pigments. All vertebrate rod pigments including bovine rhodopsin, toad red rod, anglefish red cone pigment, chicken iodopsin (54), deviate from the standard HOOP frequencies probably because of the more delocalized electronic structure needed to achieve red absorption, but the pattern of three HOOP bands is preserved. The Glu181 and Ser186 mutations do not perturb the HOOP modes, whereas the Glu113 mutations change the HOOP pattern from three bands to two at $\sim 888$ and 934 cm$^{-1}$ (53). Invertebrate octopus rhodopsin (56), which does not have a glutamic acid at the position corresponding to 113, gives the same phenotype as Glu113 mutants. This suggests that the electrostatic attraction or anchoring between the PSB and the chromophore–protein interactions have also played a role in determining the HOOP decoupling in Batho.

**Protein sequence alignments in the counterion and E2 regions reveal the residues that are required for twisting the chromophore.**
Table 1: Rhodopsin Residues in the E2 Loop, Counterion, 9-Methyl, and 13-Methyl Regions

<table>
<thead>
<tr>
<th>Residue</th>
<th>H3</th>
<th>E2</th>
<th>H5</th>
<th>H6</th>
<th>H7</th>
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<tr>
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<td>E</td>
<td>T</td>
<td>A</td>
<td>117</td>
<td>118</td>
</tr>
<tr>
<td>toad P502</td>
<td>E</td>
<td>A</td>
<td>T</td>
<td>R</td>
<td>Y</td>
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<tr>
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<td>E</td>
<td>A</td>
<td>T</td>
<td>R</td>
<td>Y</td>
</tr>
<tr>
<td>bullfrog P430</td>
<td>E</td>
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<td>T</td>
<td>R</td>
<td>F</td>
</tr>
<tr>
<td>octopus P427</td>
<td>Y</td>
<td>G</td>
<td>G</td>
<td>A</td>
<td>Y</td>
</tr>
</tbody>
</table>

a Residues < 5 Å from the 9-methyl group are bold, and residues < 5 Å from the 13-methyl group are italicized. H3, -5, -6, and -7 are rhodopsin helices. β3 and β4 are β sheets on E2.

Figure 8: Summary of the HOOP bands of the primary photoproducts in resonance Raman spectra of different visual pigments. The peak positions of HOOP modes in the Batho state are indicated for the WT bovine rhodopsin, S186I, S186A, E181D, E181Q, E113A, E113Q (53), 5-demethyl rhodopsin, 9-demethyl rhodopsin (26), octopus rhodopsin (36), toad (Bufo marinus) red rod, angelfish (Pterophyllum scalare) rods, gecko (Gekko gecko) rod, bullfrog (Rana catesbeiana) rod (54), and chicken (Gallus domesticus) iodopsin (55).

The four important residues at positions 117, 118, 188, and 189 that produce the steric barriers for the methyl groups are required to observe the Batho HOOP modes, we suggest that steric interaction of the 13-methyl group with Ala117 and of the 9-methyl group with Thr118, Gly188, and Ile189 are crucial for the distortion of the Batho chromophore. Consistently, low-temperature FTIR experiments showed that the hydroxyl group at Thr118 undergoes structural changes in the Rh–Batho transition (57). Figure 8 reveals that the electrostatic anchor between the PSB group and the Glu113 counterion works together with the steric interactions at the 9- and 13-methyl groups to provide three constraints that mediate the distortion of the ethylenic chain. When any one of these constraints is released, the twisting of the chromophore is altered as reflected by the modified HOOP mode patterns.

To determine the absolute conformation and orientation of the Batho chromophore in the binding pocket, we need to define the direction of rotation about the C11=C12 and C12=C13 bonds in the photosomerization. The initial conformation is determined by steric constraints on the 11-cis chromophore in the binding pocket, which are revealed in the crystal structure (32–34). The C12 is only 3.3 Å from the backbone oxygen of Cys187 (58), and the 13-methyl group is only 3.4 Å from the C10H group. Interestingly, steric repulsion between the 13-methyl group and C10H pushes the methyl group out of the ethylenic plane and closer to the E2 loop as shown in Figure 9A (33). To specify the absolute conformation, we use the conventional definition of the dihedral angle (59, 60). As revealed from the crystal structure, the dihedral angle about the C12–C13 bond is 150°, so the 13-methyl group is 0.8 Å above the C11=C12–C13 plane; the dihedral angle about C10–C11 is 163°, so the C10H is 0.4 Å below the C10–C11=C12 plane (see Figure 9A). To release the steric repulsions of C12 from Cys187 and of the 13-methyl group from C10H, the isomerization at C11=C12 occurs such that it rotates in a clockwise sense (viewed from lysine 296) to push the C12H down away from E2 and to bring the 13-methyl group up away from C10H. A rotation of the C11=C12 bond through 140° with a compensating reverse rotation about the C12–C13 bond through ~40° will lead to the absolute dihedral angle conformation of ~−140° at both the C11=C12 and C12=C13 bonds (Figure 9B), which is equivalent to the (~−40, ~−40) twist presented in Figure 6. Compensating distortion of the adjacent single bond, following double-bond photosomerization, was also suggested to be an important mechanism for energy storage in the primary photoevents of bacteriorhodopsin (61) and photoactive yellow protein (62).

The four important residues at positions 117, 118, 188, and 189 that produce the steric barriers for the methyl groups are located exclusively on E2 and H3. This observation is consistent with our recently proposed model, suggesting that the movement of E2 and H3 coordinated by the Cys110–Cys187 disulfide bond is crucial in the photoactivation process (19). In recent Raman studies, Pan et al. demon-
C_{12} and a $-172^\circ$ twist at the C_{12}−C_{13} bond. Their predicted same-sense dihedral twists at the C_{11}=C_{12} and C_{12}−C_{13} bonds are consistent with our Batho structure. Rohrig et al. (65) used molecular dynamics simulations to monitor the relaxation of the chromophore in the binding pocket after forcing the C_{11}=C_{12} bond to undergo cis−trans isomerization. They found that the early photoproduct is twisted in the same sense at the C_{11}=C_{12} and C_{12}−C_{13} bonds by $-161^\circ$ and $-171^\circ$, respectively. Interestingly, their photoproduct is highly twisted only when there is a salt bridge at the PSB. This result supports our three-point anchoring model, where the PSB−Glu113 salt-bridge interaction plays an essential role in twisting the ethylenic chain.

**CONCLUSIONS**

We have tested and disproved the hypothesis that Glu181 is the perturbation that causes the unique decoupled HOOP modes in Batho. Theoretical calculations are then used to support the idea that the C_{11}=C_{12} HOOP decoupling is caused by torsional distortion about both the C_{11}=C_{12} and C_{12}−C_{13} bonds in the same sense with a magnitude of $~-40^\circ$. On the basis of the crystal structure of bovine rhodopsin, we propose that the absolute dihedral angles at C_{11}=C_{12} and C_{12}−C_{13} in the Batho chromophore are both negative with values of about $-140^\circ$. Analysis of the Batho HOOP modes and of the protein sequence suggests that the distortion occurs because the chromophore is constrained by protein−chromophore interactions at three points: (1) the PSB is anchored through electrostatic interaction with the Glu113 counterion; (2) the 9-methyl group is constrained through steric interactions with Thr118, Gly188, and Ile189; and (3) the 13-methyl group is constrained through steric interaction with Ala117. Because distorted chromophores with enhanced HOOP modes are commonly observed in the primary photoproducts of photoactive proteins such as bacteriorhodopsin (2, 3), halorhodopsin (4), photoactive yellow protein (5), and phytochrome (6, 7), each of which involves rapid photoisomerization about an ethylenic bond, we anticipate that the protein-mediated distortion about the conjugated ethylenic groups that we have uncovered may be a general mechanism for energizing protein conformational changes in light-driven proteins.

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Chromophore Structure in the Primary Visual Photoprodut

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