Location of the Retinal Chromophore in the Activated State of Rhodopsin

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Rhodopsin is a highly specialized G protein-coupled receptor (GPCR) that is activated by the rapid photochemical isomerization of its covalently bound 11-cis-retinal chromophore. Using two-dimensional solid-state NMR spectroscopy, we defined the position of the retinal in the active metarhodopsin II intermediate. Distance constraints were obtained between amino acids in the retinal binding site and specific 13C-labeled sites located on the β-ionone ring, polypeptide chain, and Schiff base end of the retinal. We show that the retinal C20 methyl group rotates toward the second extracellular loop (EL2), which forms a cap on the retinal binding site in the inactive receptor. Despite the trajectory of the methyl group, we observed an increase in the C20-Gly188 (EL2) distance consistent with an increase in separation between the retinal and EL2 upon activation. NMR distance constraints showed that the β-ionone ring moves to a position between Met207 and Phe208 on transmembrane helix H5. Movement of the ring toward H5 was also reflected in increased separation between the Cα carbons of Lys296 (H7) and Met44 (H1) and between Gly121 (H3) and the retinal C18 methyl group. Helix-helix interactions involving the H3-H5 and H4-H5 interfaces were also found to change in the formation of metarhodopsin II reflecting increased retinal-protein interactions in the region of Gln122 (H3) and His211 (H5). We discuss the location of the retinal in metarhodopsin II and its interaction with sequence motifs, which are highly conserved across the pharmaceutically important class A GPCR family, with respect to the mechanism of receptor activation.

The first step in the activation mechanism of most G protein-coupled receptors (GPCRs)3 is the binding of a signaling ligand.

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3 The abbreviations used are: GPCR, G protein-coupled receptor; DARR, dipolar assisted rotational resonance; EL2, second extracellular loop; DDM, n-dodecyl-β-D-maltoside; MAS, magic angle spinning; MD, molecular dynamics; Meta, metarhodopsin; ROS, rod outer segment; 2D, two-dimensional.
4 Unless otherwise stated, the inactive, dark state of rhodopsin is referred to simply as rhodopsin, and the active intermediate is referred to as metarhodopsin II (or Meta II).
directed spin labeling and EPR measurements show that the cytoplasmic end of H6 undergoes an outward rotation upon activation (8, 9). These studies are consistent with models in which the retinal moves toward and contacts H6, placing the β-ionone ring in the H5-H6 interface. Analysis of helix-helix packing in rhodopsin indicates that the H5-H6 interface is the most loosely packed in rhodopsin (10) and may provide a low energy path for the retinal to enter/exit the retinal binding site. In fact, a recent crystal structure of opsin lacking the retinal chromophore reveals an opening in the H5-H6 interface (11).

In contrast to the idea that the retinal moves toward H6 following isomerization, studies using retinal analogs that can cross-link to the protein in the dark or following photoreaction place the ionone ring in the H4-H5 interface in Meta II. Specifically, Nakanishi and colleagues (12) found a cross-link between the β-ionone ring of retinal and Ala169 on helix H4 after illumination. Because Ala169 is on the lipid-facing surface of H4, it has been noted that a straight line drawn between Lys296 and Ala169 passes through helix H3, suggesting that this helix must consequently move upon activation (13). Additionally, a comparison of the recent 2.6 Å crystal structure of luminorhodopsin with the structure of rhodopsin reveals differences in the middle of H3 (14).

There are also proposals that the β-ionone ring does not change position upon formation of Meta II. Watts and co-workers (15) argued that the β-ionone ring anchors the retinal in position and that retinal isomerization leads to motion of H6 by inducing changes in the dihedral angles of Tyr268 and Ala269. Also, in a low-resolution crystal structure of a rhodopsin photointermediate having an unprotonated Schiff base (16), only minimal structural changes were observed relative to rhodopsin on the extracellular side of the receptor. Both models suggest that the energy stored in bathorhodopsin is largely dissipated by structural changes in helix H6 and the cytoplasmic loops.

We have previously presented solid-state NMR measurements of rhodopsin showing that there is movement of the retinal toward H5 in Meta II (17). In this study (17), the retinal was labeled with 13C at the C19 and C20 methyl groups and at the C14 and C15 carbons of the polyene chain close to the retinal protonated Schiff base linkage with Lys296. These measurements reported only on the position of the retinal methyl groups and the region of the retinal near the Schiff base and, consequently, provided only weak constraints on the final position of the β-ionone ring relative to helix H5. As a result, it was not possible to distinguish between different models that had previously been proposed for the location of the retinal in Meta II. Here, we have extended our solid-state NMR studies on rhodopsin and Meta II using retinals isotopically labeled with 13C at the retinal chain (C7, C12, C19, C20) and the β-ionone ring (C5, C6, C16, C17, and C18). These retinals are incorporated into rhodopsin containing 13C labels on different amino acids (methionine, histidine, glynine, cysteine, lysine, tyrosine, tryptophan, threonine, and phenylalanine) to define the location of the retinal in the activated Meta II intermediate. High-resolution structural constraints on rhodopsin and Meta II were established using the two-dimensional (2D) dipolar-assisted rotational resonance (DARR) NMR experiment. This approach enabled us to obtain multiple constraints by measurement of long-range 13C...13C through-space correlations between unique 13C labels. On the basis of the structural changes observed upon Meta II formation, a mechanism for the activation of rhodopsin, as well as other class A GPCRs, is proposed that involves the concerted motion of helices H5, H6, and H7 through the coupling between conserved microdomains (18, 19).

**MATERIALS AND METHODS**

**Expression and Purification of 13C-Labeled Rhodopsin—**Rhodopsin was expressed in stable tetracycline-inducible HEK293S cells (20) containing the wild-type opsin gene or mutant derivatives (21). The cells were grown in Dulbecco’s modified Eagle’s medium formulation (22) prepared from cell culture-tested components (Sigma). Fetal bovine serum was heat-inactivated before its addition to the growth medium. For suspension growth, the medium was supplemented with specific 13C-labeled amino acids (Cambridge Isotope Laboratories, Andover, MA), dialyzed fetal bovine serum (10%), Pluronic F-68 (0.1%), dextran sulfate (300 mg/liter), penicillin (100 units/ml), and streptomycin (100 μg/ml) (23, 24). The opsin gene expression was induced by addition of 2 mg/liter tetracycline and 5 mM sodium butyrate on day 5 after inoculation (20), and cells were harvested on day 7.

The HEK293S cell pellets were resuspended in phosphate-buffered saline buffer (40 ml/liter cell culture plus protease inhibitors) (21), and unlabeled 11-cis-retinal was added in two steps to a final concentration of 5 μM. The cells (40 ml/liter of cell culture) were solubilized in phosphate-buffered saline buffer plus 1% n-dodecyl-β-D-maltoside (DDM) at room temperature for 4 h. Subsequent immunoadfinity chromatography purification was carried out using the rho-1D4 antibody (National Cell Culture Center, Minneapolis, MN) as described previously (21). Column fractions containing rhodopsin were concentrated (~0.4–0.6 mM) to a final volume of ~400 μl using Centricon cones (Amicon, Bedford, MA) with a 10-kDa cutoff.

**Synthesis of 13C-Labeled Retinals and Regeneration into Rhodopsin—**Synthetic retinals with specific 13C labels were produced by standard methods (25) and purified as described previously (23). Rhodopsin pigments in DDM micelles were regenerated by illuminating concentrated samples of a 2:1 molar ratio of 13C-labeled retinal-to-protein as discussed previously (17) and concentrated to a volume of 60 μl or less by evaporation using a stream of argon gas.

**Solid-state NMR Spectroscopy—**Solid-state NMR experiments on 7–10 mg of 13C-labeled rhodopsin were performed at 14.1 teslas (600 MHz 1H frequency) on a Bruker Avance spectrometer using 4-mm magic angle spinning (MAS) probes as described previously (26). One-dimensional 13C NMR spectra were collected at MAS spinning rates between 8 and 12 kHz using ramped amplitude cross-polarization (27) with contact times of 2 ms in all experiments. 2D 13C DARR NMR measurements (28) were performed with 1024 points in the direct (f2) dimension and 64 points in the indirect (f1) dimension as described previously (26). A DARR mixing time of 600 ms was used to maximize homo-
nuclear recoupling (29). The $^1$H radiofrequency field strength during the mixing period was matched to the MAS speed for each sample, satisfying the $n = 1$ matching condition. Two-pulse phase-modulated proton decoupling (30) or SPINAL64 proton decoupling (31) was used during the acquisition and evolution periods. The decoupling field strengths were typically 80–90 kHz. To obtain the observed signal-to-noise ratios for each data set discussed below, a 2D spectrum typically required a week of signal averaging, which is approximately equal to 5120 scans for each of the 64 rows in the 1D dimension of the 2D spectrum. The carbonyl resonance of powdered glycine at 176.46 ppm relative to tetramethylsilane was used as an external $^{13}$C reference. All experiments were conducted at $-80^\circ$C.

**Trapping of the Metarhodopsin II Intermediate**—Upon light activation of rhodopsin, the active Meta II intermediate is formed within milliseconds in rod outer segment (ROS) membranes and decays with a half-time of $\sim$10 min at pH 6.0 and 20°C (32). Lowering the temperature to $-80^\circ$C blocks the decay of the Meta II intermediate; this is essential for the long periods of data acquisition required for the 2D NMR experiments. However, in ROS or model membranes one typically obtains a mixture of Meta I and Meta II after photoreaction of the retinal and low temperature trapping. To quantitatively obtains a mixture of Meta I and Meta II after photoreaction of the retinal $^{13}$C labels of the retinal that the chemical shift changes of the $^{13}$C labels of the retinal that are sensitive to the protonation state of the Schiff base nitrogen and the configuration of the C11=C12 double bond. Meta II is the only intermediate with an all-trans-retinal and an unprotonated Schiff base. We observed no residual rhodopsin or Meta I after illumination and typically were able to trap more than 85% of the sample in the Meta II intermediate, we solubilized rhodopsin in DDM detergent.

Samples were irradiated for 45–60 s at room temperature in the NMR rotor using a 400-watt lamp equipped with a greater than 495-nm long pass filter and immediately put in the NMR probe with the probe stator warmed to 5°C. Under slow spinning ($\sim$2 kHz) the sample was frozen within 3 min of illumination using $N_2$ gas cooled to $-80^\circ$C (17).

To confirm that we had trapped Meta II and completely converted the sample from rhodopsin and Meta I, we monitored the chemical shift changes of the $^{13}$C labels of the retinal that are sensitive to the protonation state of the Schiff base nitrogen and the configuration of the C11=C12 double bond. Meta II is the only intermediate with an all-trans-retinal and an unprotonated Schiff base. We observed no residual rhodopsin or Meta I after illumination and typically were able to trap more than 85% of our original sample in the Meta II state based on integration of the retinal $^{13}$C resonances in rhodopsin and Meta II (17).

The line widths of the resolved protein and retinal NMR resonances are generally between 1 and 2 ppm in both rhodopsin and Meta II. The absence of line broadening or resonance splitting indicated that we had trapped a spectroscopically well-defined Meta II state. Molecular dynamics (MD) simulations and $^2$H NMR measurements on rhodopsin have suggested that there are two major conformations of the $\beta$-ionone ring, one with a positively twisted C6—C7 single bond and one with a negatively twisted C6—C7 single bond (33, 34). As described below, $^{13}$C chemical shift and DARR NMR measurements suggest that, predominantly, a single conformation exists in rhodopsin and in Meta II.

Meta II in DDM has been found to be functionally equivalent to Meta II in ROS membranes as it can activate transducin (35, 36). Also, it has been shown that the vibrational frequencies observed in the Fourier transform infrared difference spectrum of Meta II minus rhodopsin are identical for rhodopsin in DDM and ROS membranes (37, 38). The mean half-time for proton uptake of Meta II in DDM is 25 ms (39). As mentioned above, the time between illumination and freezing of the sample is $\sim$3 min, indicating that the proton uptake in our sample is complete and that we were observing the form of Meta II that activates transducin.

**RESULTS**

**Location of the $\beta$-Ionone Ring Relative to H5 in Metarhodopsin II**—Our previous solid-state NMR studies concluded that the retinal moves toward H5 in the rhodopsin-to-Meta II transition (17). We sought to establish whether the $\beta$-ionone ring increases its contact with H5 by targeting the terminal $\epsilon$-methyl group of Met$^{207}$, which is on the face of H5 oriented toward the $\beta$-ionone ring of the retinal. For these experiments, rhodopsin containing $^{13}$C$\varepsilon$-labeled methionine was regeneranted with 11-cis-retinal labeled with $^{13}$C at the C6 and C7 carbons (Fig. 1A).

![FIGURE 1. Structure of the retinal binding site in rhodopsin. A, structures of the 11-cis- and all-trans-retinal chromophores. B, view of the rhodopsin crystal structure (Protein Data Bank code: 1U19) from the extracellular surface showing transmembrane helices H3-H7 and the position of key amino acids discussed in the text. C, view along the axis of the retinal from the perspective of H5.](image)

Fig. 2A presents the 2D DARR NMR spectra of rhodopsin showing only the region containing cross-peaks between the 13C6 and 13C7 retinal resonances and the 13C resonance of methionine. The rhodopsin spectrum (Fig. 2A, black) exhibits a weak cross-peak between the 13C7 resonance of the retinal chromophore and Met$^{207}$ (14.7 ppm). The weak intensity of the Met$^{207}$-C7 cross-peak (Fig. 2A) is consistent with the separation of 4.9 Å observed in the rhodopsin crystal structure (40). No cross-peaks are observed between the 13C6 resonance of Met$^{207}$ and the 13C6 resonance of the retinal; the 5.4 Å distance between these 13C labels is near the upper limit of the distance range of the DARR experiment (29). Upon conversion to Meta II (Fig. 2A, red) both the 13C6 and 13C7 resonances exhibit strong cross-peaks with the 13C-labeled Met$^{207}$ resonance at 13.8 ppm. The intensity of the Meta II cross-peaks indicates that the C6 and C7 carbons are in van der Waals contact with Met$^{207}$.

NMR measurements were also made on rhodopsin containing [13C$\varepsilon$]methionine and regeneranted with [13C5,13C18]-labeled retinal. In the DARR NMR spectra of rhodopsin, cross-peaks were not observed between Met$^{207}$ and either the 13C5 or 13C18...
resonances of the retinal β-ionone ring. In the rhodopsin crystal structure (40), the Met207(Ce)-retinal C5 (6.7 Å) and the Met207(Ce)-retinal C18 (7.7 Å) distances are outside the range of the DARR NMR experiment. However, upon conversion to Meta II, we observed strong cross-peaks between Met207 and both the 13C5 (13.8 ppm) and 13C18 (20.9 ppm) resonances of the retinal.

The Met207(Ce)/C6/C7/C18 contacts are consistent with our previous observation of movement of the retinal toward H5 (17). However, the data do not tightly constrain the location of the ionone ring relative to H5 because of the flexibility of the long methionine side chain. To better establish the position of the ionone ring relative to H5 in Meta II, additional 13C–13C recoupling experiments involving the retinal and several amino acids in the H4–H5 and H5–H6 interfaces were undertaken.

To address the trajectory of the retinal suggested by retinal cross-linking to Ala169 on helix H4 (12), we obtained 2D DARR NMR spectra of rhodopsin 13C-labeled at the Cβ carbon of Cys167 and at the C5, C6, and C7 carbons of retinal. On the basis of molecular modeling of the retinal binding site, Cys167 should be in close proximity to one of these carbons if the retinal adopts the position in the H4–H5 interface suggested by cross-linking to Ala169. In rhodopsin, the Cβ carbon of Cys167 and the C5—C7 carbons are separated by >7.8 Å. In the 2D DARR NMR spectrum of rhodopsin, no cysteine-retinal cross-peaks were observed (data not shown). Similarly, in the 2D DARR NMR spectrum of Meta II we were unable to detect cysteine-retinal cross-peaks, arguing that the retinal C5—C7 carbons are more than 6 Å from the Cβ carbon of Cys167 (data not shown).

To observe contacts between the retinal chromophore and phenylalanine in the H5–H6 interface, we 13C-labeled the C16 and C17 methyl groups on the retinal β-ionone ring, as well as the ring carbons of phenylalanine. The 13C resonances of the aromatic ring of phenylalanine at 120–145 ppm are well resolved from the 13C resonances of the C16 and C17 retinal methyl groups between 25 and 33 ppm. Fig. 3A presents the one-dimensional MAS difference spectrum between rhodopsin (positive) and Meta II (negative) in the region of the retinal methyl resonances. The positive peaks at 30.6 and 26.1 ppm have previously been assigned to the 13C16 and 13C17 resonances of the retinal methyl groups in rhodopsin (41), respectively. On the basis of IUPAC nomenclature, the C1—C16 bond is oriented into the plane of the page in Fig. 1A, whereas the C1—C17 bond is oriented out of the plane of the page. The difference between the 13C chemical shifts (4.5 ppm) of the C16 and C17 resonances is attributed to one methyl group being in an equatorial orientation and the other methyl group being in an axial orientation. A steric contact between the methyl group in the axial orientation and the proton on C3 of the β-ionone ring results in an upfield chemical shift (41, 42). The negative peaks at 28.6 and 33.4 ppm correspond to the chemical shifts of the C16 and C17 methyl groups in Meta II, respectively (data not shown). The difference in chemical shift (4.8 ppm) between the 13C16 and 13C17 resonances is again consistent with a difference in the relative orientation of the methyl groups. However, the shift of C16 from 30.6 to 28.6 ppm and of C17 from 26.1 to 33.4 ppm indicates that C16 has now moved to the axial position in Meta II.

Fig. 3, B and C, presents rows extracted from the 2D DARR NMR spectra of rhodopsin containing 13C-ring-labeled phenylalanine and regenerated with 11-cis-retinal 13C-labeled at the
C16 and C17 positions. The rows are taken through the $^{13}$C resonance of the aromatic phenylalanine ring on the diagonal of the 2D spectrum. Vertical lines drawn from the MAS difference spectrum indicate the positions of the retinal-phenylalanine cross-peaks. There are 31 phenylalanines in rhodopsin. In the rhodopsin crystal structure, the closest ring carbons of Phe$^{208}$ are 4.2 Å from C17 and 4.7 Å from C16. Phe$^{212}$ is the only other phenylalanine within 7 Å of the C16 and C17 methyl groups. Therefore, the contacts detected between phenylalanine and the $^{13}$C16 and $^{13}$C17 resonances at 30.6 and 26.1 ppm, respectively, in Fig. 3B are assigned to Phe$^{208}$ and/or Phe$^{212}$. Although the 4.2 and 4.7 Å distances are within the range of the DARR experiment, the low signal intensity (compared with an isolated pair of $^{13}$C spins separated by the same distance) is due to dipolar truncation (43), which makes it difficult to measure a weak dipolar coupling in the presence of a strongly coupled network of spins.

Upon conversion to Meta II, the cross-peaks assigned to retinal-phenylalanine contacts in rhodopsin disappear. New cross-peaks of similar intensity appear at the chemical shifts assigned to the $^{13}$C16 and $^{13}$C17 resonances in Meta II (Fig. 3C). These contacts are also assigned to Phe$^{208}$ and/or Phe$^{212}$ based on the proximity of the retinal to Met$^{207}$ discussed above. The similar intensities of the C16,C17-phenylalanine cross-peaks in the inactive and active states are consistent with the $\beta$-ionone ring maintaining an approximate distance of 4–5 Å from Phe$^{208}$ and/or Phe$^{212}$ in Meta II.

To further characterize the position of the retinal, DARR NMR measurements were made between the retinal $^{13}$C16- and $^{13}$C17-labeled methyl groups and the backbone carbonyls of Met$^{207}$ ($^{13}$C1) and His$^{211}$ ($^{13}$C1). Fig. 3D presents the 2D DARR NMR spectra of rhodopsin (black) and Meta II (red) containing $^{13}$C1 histidine and $^{13}$C16, $^{13}$C17-labeled retinal. His$^{211}$ is the only histidine within 15 Å of the C16 and C17 methyl groups in rhodopsin. The $^{13}$C1 histidine chemical shifts for His$^{211}$ in rhodopsin (172.4 ppm) and Meta II (170.2 ppm) agree with those assigned previously to His$^{211}$ (44). Fig. 3E presents the 2D DARR NMR spectra of rhodopsin (black) and Meta II (red) containing methionine $^{13}$C-labeled at the carbonyl position and regenerated with $^{13}$C16-, $^{13}$C17-labeled retinal. The Met-retinal cross-peaks in these spectra are assigned to Met$^{207}$ based on the close proximity of these groups in rhodopsin. Although $^{13}$C1 histidine and $^{13}$C16, $^{13}$C17-retinal cross-peaks are observed in both rhodopsin and Meta II, an increase of intensity upon conversion to Meta II is consistent with tighter packing of the $\beta$-ionone ring of retinal with the backbone of H5.

Location of the $\beta$-I onone Ring Relative to H3 and H6 in Metarhodopsin II—In the crystal structure of rhodopsin, the 11-cis-retinal chromophore is tightly packed in the protein interior (Fig. 1, B and C). As noted in the introduction, the all-trans isomer of retinal cannot fit in the binding site of the inactive state of rhodopsin, indicating that the shape of the binding cavity must change upon activation. The results described above and in our previous studies (17) are consistent with motion of the retinal chromophore toward H5. In this section, $^{13}$C DARR NMR measurements are described for rhodopsin $^{13}$C-labeled at Thr$^{118}$ (H3), Gly$^{121}$ (H3), and Trp$^{265}$ (H6) and regenerated with 11-cis-retinal $^{13}$C-labeled at the C18 and C19 methyl groups to further address the motion of the retinal $\beta$-ionone ring relative to H3 and H6.

Fig. 4A shows the positions of Gly$^{121}$ and Trp$^{265}$ and the C18 methyl group on the retinal $\beta$-ionone ring. In the rhodopsin crystal structure (40), the retinal C18 methyl group is at 3.7 Å from the Cα carbon of Gly$^{121}$. In the 2D DARR NMR spectrum, we observe a strong cross-peak (highlighted by a green box) between the 13C resonance of Gly$^{121}$ on H3 and the retinal 13C18 methyl group, consistent with a separation of 3.7 Å (Protein Data Bank code: 1U19). This contact is lost upon conversion to Meta II. C, region of Trp$^{265}$. Retinal C18 contacts. In rhodopsin, a weak contact is observed between the ring carbons of [U-$^{13}$C]Trp$^{265}$ and the retinal 13C18 methyl group. This contact is lost in Meta II. D, region of Thr$^{118}$-retinal C19 contacts. In rhodopsin, a cross-peak between [U-$^{13}$C]Thr$^{118}$ on H3 and the retinal 13C19 methyl group overlaps with the intense intra-residue cross-peaks of [U-$^{13}$C]Thr$^{118}$. The cross-peak is more clearly resolved in Meta II (red) where the 13C19 resonance has shifted to a lower frequency. The observation in Meta II of a retinal C19-Thr$^{118}$ cross-peak of roughly the same intensity as in rhodopsin indicates that the retinal-Thr$^{118}$ distance does not change considerably upon activation.
ulations on rhodopsin, Lau et al. (34) observed two distinct conformations, one conformation with a negative twist about the C6—C7 single bond, placing the C18 methyl group near Gly121 (as observed) and one with a positive twist placing the C18 methyl group near Tyr268. DARR NMR experiments on [13C]tyrosine-labeled rhodopsin containing 13C18-labeled retinal failed to exhibit a C18-Tyr cross-peak (data not shown), indicating that if present the positively twisted conformer is only a minor component.

In the rhodopsin crystal structure (40), the side chain of the highly conserved Trp265 (H6) is tightly packed against the β-ionone ring of the retinal. The retinal C18 methyl group is at 3.7 Å from the nearest indole ring carbon. To test whether the retinal C18 methyl group remains in contact with Trp265 after conversion to Meta II, we obtained 2D DARR NMR spectra of rhodopsin and Meta II containing [U-13C]tryptophan, [13C]glycine, and [13C5,13C18]retinal. We observed a weak cross-peak between the aromatic ring carbons of Trp265 and the C18 methyl group in rhodopsin (Fig. 4C). The cross-peak was not observed in Meta II, consistent with an increase in the distance between the β-ionone ring and Trp265. We attribute the weak intensity of the C18-Trp265 cross-peak in rhodopsin to dipolar truncation (43), a problem encountered in the measurement of long internuclear distances (i.e. weak dipolar couplings) using uniformly 13C-labeled amino acids (i.e. in the presence of strongly 13C-coupled networks).

To assess the contribution of dipolar truncation to the observed intensities in rhodopsin containing [U-13C]tryptophan, [13C]glycine, and [13C5,13C18]retinal, DARR NMR experiments were run on a parallel rhodopsin sample containing only [13C]glycine and [13C5,13C18]retinal. Fig. 5, A and B, correspond to rows through the retinal C5 diagonal at 131.0 ppm of two rhodopsin samples. The cross-peak at 21.6 ppm arising from the directly bonded C5—C18 carbons is significantly increased in intensity when Trp265 is not 13C-labeled, consistent with dipolar truncation being the cause of the intensity loss in our measurement of the retinal C18-Trp265 contact. In contrast, on conversion to Meta II the intensity of the 13C5—13C18 cross-peak (measured through the C5 diagonal at 126.0 ppm) in the sample labeled with [U-13C]tryptophan (Fig. 5C) is comparable with the intensity of the 13C5—13C18 cross-peak in the sample containing unlabeled tryptophan (Fig. 5D), suggesting that Trp265 (H6) and C5—C18 have moved away (>6 Å) from each other in Meta II. As a result, the difference in intensity of the 13C5—13C18 cross-peak in Fig. 5, A and C, is consistent with an increase in separation between the retinal C18 methyl group and Trp265 in Meta II.

The retinal C18-Gly121 and retinal C19-Trp265 distance constraints described above complement previous Gly121, Trp265, and retinal C19-Trp265 distance measurements in which we observed the loss of a Gly121-Trp265 contact and the gain of a retinal C19-Trp265 contact in Meta II (26). These earlier results were interpreted in terms of rotation or translation of the Trp265 side chain toward the extracellular side of the retinal binding site upon formation of Meta II.

The cross-peak between the retinal C19 methyl group and Trp265 provides a point of contact between the retinal chromophore and H6 in Meta II. To obtain a corresponding point of contact with H3, we undertook DARR NMR measurements of rhodopsin containing 13C19-labeled retinal and [U-13C]tyrosine (Fig. 4A). In the rhodopsin crystal structure (40), the retinal is packed against Ala117, Thr118, and Glu122 on H3. Thr118 is adjacent to the middle of the retinal polype chain and is the only amino acid among these three that can be 13C-labeled in our HEK293S expression system. The retinal C19 methyl group is one of the closest points of contact with H3 and is within 5 Å of Thr118. The next closest threonine is over 10 Å away. In Meta II, the cross-peak between the 13C19 methyl group and the unresolved 13Cα and 13Cβ carbons of Thr118 does not change appreciably in intensity, indicating that the retinal C19 methyl group maintains contact with H3 at this position (Fig. 4D).

Location of the Polynene Chain in Metarhodopsin II—In our earlier work (17), the conclusion that the retinal moves toward H5 was based largely on the loss of cross-peaks between the retinal C19 methyl group and the C5 carbons of Tyr191 (EL2) and Tyr268 (H6), as well as on the gain of a tyrosine contact with the retinal C20 methyl group that we had tentatively assigned to Tyr179 (EL2). To conclusively assign the C20-Tyr contact in Meta II, we obtained DARR NMR spectra of the Y178F rhodopsin mutant labeled with [13C]tyrosine and regenerated with [13C12,13C20]retinal.

Fig. 6 presents the DARR NMR spectra of wild-type rhodopsin (left) and the Y178F mutant of rhodopsin (right). Fig. 6 presents rows through the diagonal resonance of tyrosine at ~156 ppm in both the dark state of rhodopsin (black) and the Meta II intermediate (red); the upper panels (A and C) show the region containing retinal C12-tyrosine cross-peaks, and the
Retinal Location in Metarhodopsin II

FIGURE 6. 2D DARR NMR of wild-type rhodopsin and the Y178F mutant labeled with [13C]glycine and regenerated with [13C12, 13C20]retinal. A and B, rows are shown taken through the [13C]Tyr diagonal from the DARR NMR spectra of wild-type rhodopsin (black) and Y178F mutant of rhodopsin (red). The region containing cross-peaks to the retinal 13C12 carbon is shown in A and to the retinal 13C20 carbon in B. C and D, the same regions of the DARR NMR spectra of the Y178F mutant of rhodopsin (black) and Meta II (red) are taken through the [13C]Tyr diagonal. Asterisks indicate MAS side bands.

lower panels (B and D) show the region containing the C20-Tyr cross-peaks. In rhodopsin (Fig. 6, black spectra), we observe cross-peaks between Tyr268 and both the C12 and C20 resonances. The 13C-Carbon of Tyr268 is close to both C12 (4.9 Å) and C20 (4.2 Å) in the inactive state of rhodopsin (40).

In Fig. 6, B and D, we observe a single retinal-tyrosine cross-peak in both wild-type Meta II and the Y178F mutant of Meta II (red spectra). The observation of a retinal-tyrosine cross-peak in the Y178F mutant rules out Tyr178 as the tyrosine having a contact to the C20 methyl group (viewed from the Schiff base end of the retinal) of the C20 methyl group agrees with the crystal structure of rhodopsin (40). However, these cross-peaks are lost in Meta II, indicating that the C12-C13-C20 plane is oriented such that the C20 methyl group is closer to Gly114 on the extracellular side of the binding site, and C12 is pointing away from Gly114 (H3) toward the cytoplasmic side of the binding site. MD simulations (see supplemental material), using the C20-Gly114 assignment, produce a model of the all-trans-retinal in Meta II with the C13-C20 bond oriented toward H3 at an angle of ~60° to the membrane normal. Independently, site-directed deuteron NMR studies on Meta I by Brown and co-workers (45) have shown that the C13-C20 bond is oriented at an angle of 59 ± 3°. Although the deuteron NMR studies are on Meta I, they support the assignment of the C20-Gly114 contact in Meta II.

Second, the assignment suggests that EL2 moves away from the retinal chromophore upon activation. The large rotation of the C20 methyl group agrees with the crystal structure of bathorhodopsin (46, 47), which reveals a clockwise rotation of the C20 methyl group (viewed from the Schiff base end of the retinal) and a number of computational (46, 48) and biophysical (45) studies. Such a rotation would place the C20 methyl group close to Gly114 on EL2 in the absence of EL2 motion. We had previously assumed that the position of EL2 did not change in the formation of Meta II (17) because of the network of hydrogen bonding interactions involving EL2 and the extracellular ends of the transmembrane helices. These new data challenge this assumption. Moreover, recent DARR NMR distance measurements (49) show that contacts between the retinal chromophore and the β4 strand of EL2 are lost in Meta II.

Third, the assignment of the C20-Gly contact to Gly114 in Meta II suggests that the C20-tyrosine contact is with Tyr268 rather than with Tyr191. In rhodopsin, the C20 methyl group has contacts with Tyr268 and Trp265, whereas the C19 methyl group has contacts with Tyr191 and Tyr268 (17). In Meta II, there...
FIGURE 8. 2D DARR NMR of rhodopsin and Meta II labeled with \[^{13}C\]methionine and \[^{13}C\]lysine. A, rows through the 2D DARR NMR spectra of rhodopsin (black) and Meta II (red) taken through the Met207 (H1) diagonal resonance at 10.5 ppm showing the cross-peak with \[^{13}C\]Lys296 (H7) at 49.1 ppm. On conversion to Meta II (red) the cross-peak becomes weaker consistent with an increase in separation between Met44 (19.4 ppm) and Lys296 (60.8 ppm). B, view of the retinal binding site in rhodopsin (Protein Data Bank code: 1U19) from the extracellular side of the protein highlighting the orientation of the retinal with respect to specific residues on H1 (Met44), H3 (Thr118), H5 (Met207), H6 (Trp265), and H7 (Lys296).

are no tyrosine cross-peaks associated with C19, and as noted, we observed only a single tyrosine cross-peak to C20. We had previously argued for a 4–5 Å translation of the retinal toward H5 in order to move the C19 methyl group away from Tyr191 and Tyr268. The distance constraints presented here and in a manuscript on the motion of EL2 published elsewhere (49) indicate that the translation of the retinal is more modest (∼2 Å) and that EL2 moves away from the retinal upon activation. Motion of EL2 away from the retinal chromophore would increase both the C19–Tyr191 distance and the C20–Tyr191 distance, leaving only Tyr268 in relatively close proximity to the C20 methyl group. We have not been able to confirm this assignment by mutational studies because mutation of Tyr268 to phenylalanine appears to alter its interactions with EL2 (49).

Location of the Retinal Schiff Base in Metarhodopsin II—The DARR NMR measurements described above between the \[^{13}C\]methionine and \[^{13}C\]lysine argues for a small (∼2 Å) shift of the retinal toward H5. To obtain further support for motion of the retinal toward H5, we also measured the distance between the \[^{13}C\]carbon of Lys296 on H7 and the \[^{13}C\]carbon of Met44 on H1. The \[^{13}C\]Met44-to-\[^{13}C\]Lys296 distance in rhodopsin is 4.7 Å. Fig. 8A presents rows through the \[^{13}C\]diagonal resonance of methionine in the DARR NMR spectrum of rhodopsin (black) and Meta II (red). The Met44-Lys296 cross-peak intensity decreases in the conversion to Meta II consistent with an increase in the internuclear distance.

Rearrangement of H3-H4-H5 Contacts in Metarhodopsin II—In this section, we describe additional solid-state NMR measurements to address the impact of retinal movement on the position of helix H5. Solvent accessibility studies (50) and recent double electron-electron resonance EPR measurements (9) suggest that the cytoplasmic end of H5 does not undergo large, rigid body motion as observed for H6. However, we have recently shown that a hydrogen bond between His211 on H5 and Glu122 on H3 is broken in Meta II (44), suggesting a change in helix-helix packing and H5 motion. Below, we take advantage of the known chemical shifts of His211 and Met207 to probe how the H4-H5 interface changes in Meta II.

Fig. 9 shows the 2D DARR NMR spectra of rhodopsin (black) and of Meta II (red) labeled with \[^{13}C\]methionine, \[^{13}C\]methionine, and \[^{13}C\]histidine. A, Cys-Met contacts in rhodopsin (black) and Meta II (red). Above the 2D spectrum are rows taken through the Met207 diagonal. A cross-peak with Cys167 at 25.3 ppm appears in Meta II. B, His-Cys and His-Met contacts in rhodopsin and Meta II. Above the 2D spectrum are rows taken through the His211 diagonal in rhodopsin (136.9 ppm) and Meta II (137.5 ppm). In rhodopsin, cross-peaks are observed with Cys167 at 23.7 ppm and Met163 at 13.1 ppm. In Meta II, cross-peaks are observed with Cys167 at 25.3 ppm, Met207 at 13.8 ppm, and Met163 at 11.2 ppm.
Retinal Location in Metarhodopsin II

The broad His211–Met cross-peak at 13.1 ppm is consequently assigned to Met207.

In Meta II (Fig. 9B, red), the His211–Cys167 cross-peak loses intensity and shifts to 25.3 ppm. The assignment to Cys167 can be made because it is the only cysteine within 17 Å of His211. The decrease in intensity of the histidine-cysteine cross-peak indicates that there is an increase in the His211–Cys167 distance.

The His–Met region of the 2D DARR NMR spectrum in Fig. 9B also places constraints on the position of H5 in Meta II; two cross-peaks (red) are observed between His211 and methionine in Meta II. There are only two methionines within 16 Å of His211 in rhodopsin. The cross-peak at 13.8 ppm is assigned to Met207 based on the chemical shift of the cross-peak observed between Met207 and the retinal 13C6,13C7 carbons (Fig. 2A). The cross-peak at 11.2 ppm is assigned to Met163. These data indicate that the terminal methyl groups of both Met163 and Met207 are within 6.0 Å of His211.

The multiple contacts observed for Met207 in Meta II provide strong constraints on its location. The Met207 side chain must be positioned between Cys167 and the retinal because we did not observe a retinal-Cys167 cross-peak. The chemical shift of Met207 undergoes a small (0.9 ppm) shift in frequency upon conversion from rhodopsin to Meta II. The unique shift and narrow line width indicate that the Met207 side chain is not disordered. The decrease in intensity of the His211–Cys167 cross-peak and the appearance of a new Met207–Cys167 contact in Meta II are consistent with motion of the extracellular end of the H5 helix.

Recently, Watts and co-workers (15) have argued that the position of the β-ionone ring does not change upon the formation of Meta II on the basis of an analysis of the 13C chemical shifts of the C16 and C17 methyl groups. This conclusion is essentially correct relative to the large translation proposed by Nakanishi and co-workers (12). However, the small, but significant, changes we observed, which are in disagreement with the detailed model proposed on the basis of an immobile ionone ring (15), are critical for the motions of H5 and H6, as discussed below. Changes in the chemical shifts observed due to sample illumination in the previous study (15) (~0.7 ppm for C17) are smaller than those observed here (2–3 ppm for both C16 and C17). The differences can arise from incomplete trapping of Meta II or differences in the Meta II intermediate trapped in lipid and detergent environments. We (51, 52) and others (37, 53) find that illumination of rhodopsin in DDM detergent results in nearly quantitative conversion (> 85%) to Meta II, whereas illumination of rhodopsin in unsaturated lipids and ROS membranes results in a mixture of intermediates containing both protonated and unprotonated retinal Schiff bases at pH 7.0 (54).

DISCUSSION

We have previously presented solid-state NMR measurements of rhodopsin showing that there is movement of the retinal toward H5 in Meta II (17). These measurements only probed the position of the retinal C19 and C20 methyl groups and the region of the retinal near the Schiff base and, consequently, provided only weak constraints on the final position of the β-ionone ring relative to helix H5. As a result, it was not possible to distinguish between different models that had previously been proposed for the location of the retinal in Meta II (12, 15). In this manuscript, we provide additional structural constraints for the location of the retinal in the activated Meta II intermediate and consider their implications on the structural changes that occur upon conversion of rhodopsin to Meta II.

Retinal-H3 and Retinal-H5 Interactions—The cross-peaks observed between the 13C labels on the retinal and histidine, phenylalanine, and methionine on H5 tightly constrain the position of the β-ionone ring between Met207 and Phe208 in Meta II. Moreover, the observation of a new Met207–Cys167 contact and the lack of a contact between the retinal and Cys167 in Meta II rule out the possibility that the β-ionone ring is located in the H4–H5 interface. These data argue that the position of the retinal derivative used in the cross-linking studies of Nakanishi and co-workers (12) is different than in wild-type Meta II.

A structural model resulting from MD simulations guided by our NMR data (see supplemental material) suggests an explanation for the role of the β-ionone ring in the stability of Meta II (Fig. 10). The crystal structure of rhodopsin shows a hydrogen...
bonding contact between the side chain of Glu^{122} on H3 and the backbone carbonyl of His^{211} on H5. The His^{211} carbonyl does not participate in main chain hydrogen bonding because of the highly conserved Pro^{215} at the i+4 position. In the Meta II model derived from guided MD simulations, the β-ionone ring contacts the side chain of Glu^{122} on H3 as well as the backbone of H5 near His^{211}. The hydrogen bond between the main chain carbonyl of His^{211} and the side chain of Glu^{122} is disrupted as observed previously by solid-state NMR (44). The position of H5 allows the formation of a new hydrogen bond between Glu^{122} and the His^{211} δ-nitrogen, which stabilizes the Meta II structure.

The role of Glu^{122} and His^{211} in stabilizing the Meta II state is supported by comparative studies between rhodopsin and the cone pigments, which lack these residues. Shichida and co-workers (55) found that substitution of Glu^{122} in rhodopsin with the corresponding amino acid in the green- or red-sensitive cone pigments converts the rate of retinal regeneration and Meta II decay into those characteristic of the respective cone pigments. In contrast, when glutamate is substituted into the green-sensitive cone pigment, the rates of retinal regeneration and Meta II decay are similar to those of rhodopsin.

H5 in many other class A GPCRs has been shown to interact with receptor-specific ligands. For instance, in the amine receptors (such as the β₂-adrenergic receptors), the amino acids corresponding to Met^{207}, Phe^{208} and His^{211} in rhodopsin are conserved as serines. These serines hydrogen bond to hydroxylo groups on the catechol ring of the amine agonist to stabilize an active receptor conformation (56–58). In the dopamine (59) and serotonin (60) receptors, the amino acids at the positions equivalent to His^{211} and Phe^{212} have been shown to interact with their receptor-specific ligands.

The recent crystal structure of opsin (11), which is formed following the release of all-trans-retinal in Meta II, shows that the position of the extracellular end of helix H5 is the same as in rhodopsin, although some of the side chain interactions predicted for Meta II are retained. For example, the backbone carbonyl of His^{211} (H5) is no longer hydrogen bonded to the Glu^{122} (H3) side chain. Instead a new interaction is observed directly between the Glu^{122} (H3) and His^{211} (H5) side chains consistent with our Meta II data. Also, Trp^{265} (H3) has lost its hydrogen bonding interaction with Glu^{122} (H3) in opsin and appears to be hydrogen bonded only to His^{211} on H5. A weakening of the hydrogen bonding interaction for Trp^{265} was observed in Meta II by NMR (26) and UV-visible absorption spectroscopy (61). However, the hydrogen bonding interactions for Tyr^{206} in opsin are not consistent with recent NMR results showing that Tyr^{206} undergoes a significant weakening of hydrogen bonding in Meta II (49). Also, the distance constraints obtained from our NMR experiments for pairs of amino acids in the H3-H4-H5 interface in Meta II, such as His^{211}(H5)-Cys^{167}(H4), His^{211}(H5)-Met^{160}(H4), His^{211}(H5)-Met^{207}(H5), and Met^{207}(H5)-Cys^{167}(H4), are not consistent with the arrangement of residues in the opsin crystal structure. As a result, it appears that the all-trans-retinal chromophore holds helix H5 in an active conformation and that loss of the retinal allows the receptor to shift (at least partially) back to an inactive conformation. These changes are in agreement with retinal analog studies showing the importance of the β-ionone ring in receptor activation (62).

In contrast to the inactive conformation of the extracellular end of H5, the cytoplasmic end of H5 in the opsin crystal structure (11) appears to adopt the “active” conformation of the receptor. The cytoplasmic end of H5 has moved inward and rotated to place conserved Tyr^{223} in contact with Arg^{135}, breaking the ionic lock between Arg^{135} on H3 and Glu^{247} on H6. The NMR results showing a movement of H5 due to ionic interactions with the ionone ring and coupling with EL2 (49) are consistent with the key role of H5 motion in activation.

**Retinal-H6 Interactions**—An aromatic cluster of amino acids at the extracellular end of H6 represents a functional microdomain that is key to receptor activation in the class A GPCRs. In rhodopsin, Phe^{261}, Trp^{265}, and Tyr^{268} on H6 form this cluster. The aromatic side chain of Trp^{265} lies in the arc created by the 11-cis-retinal and the side chain of Lys^{296}. We have previously proposed that preventing motion of Trp^{265} locks the receptor off by inhibiting the motion of H6 (26). Motion of the β-ionone ring toward H5 in the rhodopsin-to-Meta II transition allows motion of the Trp^{265} side chain or the H6 backbone toward EL2. Previously, we observed that Trp^{265} comes into close contact with the retinal C19 methyl group in Meta II (26) and loses its contact with Gly^{121} on H3. Here, we report that the C18 methyl group of the β-ionone ring moves away from Trp^{265} and Gly^{121}. In the guided MD simulations, to satisfy the NMR constraints (see supplemental Table I) the Trp^{265} side chain undergoes significant motion toward EL2 while remaining in van der Waals contact with the C19 methyl group on the retinal polyene chain. In the opsin crystal structure (11), the indole side chain of Trp^{265} has moved away from Gly^{121} and Ala^{296} consistent with the NMR data but has not undergone a change in rotameric state or shifted toward EL2 to any significant extent. We attribute the differences between the opsin structure and our model of Meta II to the lack of all-trans-retinal in the binding cavity.

Our conclusions regarding retinal-H6 interactions are in agreement with a wide range of previous studies on both rhodopsin and other GPCRs. For instance, UV absorbance and linear dichroism studies on rhodopsin show that Trp^{265} moves into a more hydrophilic environment and that the plane of the indole side chain of Trp^{265} changes from an orientation parallel to the bilayer normal to an orientation roughly perpendicular to the bilayer normal (61, 63). Javich and co-workers (64) proposed a “rotamer toggle switch” for receptor activation based on studies of the β2 adrenergic receptor, in the subclass of biogenic amine receptors. In these receptors, ligand binding is thought to induce a change in the side chain conformation of a conserved phenylalanine, which is coupled to changes in the side chain conformations of conserved cysteine, tryptophan, and proline residues on H6. The location of Trp^{265} in opsin is consistent with the linear dichroism studies in which rotation of a tryptophan in the Meta I to Meta II transition was found to be reversed upon decay of Meta II (63).

**Retinal-H7 and Retinal-EL2 Interactions**—The extracellular and cytoplasmic ends of H7 are both important in the activation mechanism of rhodopsin (17). The extracellular end of H7 contains residues that are specific to the subfamily of class A

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GPCRs (17, 65). For example, in rhodopsin the retinal is attached to Lys296 (H7) as a positively charged protonated Schiff base that forms a salt bridge with the negatively charged side chain of Glu113 (H3). This interaction is important for keeping the receptor in the inactive state and is broken upon receptor activation (66).

The cytoplasmic end contains the highly conserved NP.VXY sequence. The recent crystal structures of opsin shows that Tyr306 is reoriented toward Arg135 of the ionic lock (11, 67), which is stabilized in an open conformation by elements from H5 (Tyr221), H6 (Met257), and H7 (Tyr306). Together, these observations indicate that retinal isomerization (or ligand binding in the ligand-activated GPCRs) results in structural changes involving the extracellular end of H7 that allow the cytoplasmic end to adopt an active conformation.

How does retinal isomerization and Schiff base deprotonation lead to motion of H7? There are two possibilities. The first possibility is that a change in the position of the retinal directly affects the position of H7 through its covalent linkage to Lys296. We previously proposed a “push-pull” mechanism for activation involving a push on H5 by the ionone ring and a pull on H7 via the covalently attached retinal (17). As mentioned above, the distance constraints presented here and in a manuscript on the motion of EL2 published elsewhere (49) indicate that translation of the retinal toward H5 is more modest (2 Å) and that EL2 moves away from the retinal binding site upon activation. The reduced motion of retinal toward H5 described above would suggest a more modest role for the direct covalent bond between the retinal and H7. In fact, Oprian and co-workers (68) have shown that a covalent bond between retinal and Lys296 is not required for activation.

The second and more likely possibility for how retinal isomerization and Schiff base deprotonation lead to a change in the position of the extracellular end of H7 is that the H7 helix responds to changes in other structural elements in the protein. The closest elements that change position in Meta II are EL2 and H6. The extracellular end of H7 between Met288 and Ala292 packs against EL2 and will likely shift due to displacement of EL2 as a result of changes in both steric and electrostatic interactions. H6 packs against H7 along its entire length, and mutations along the H6-H7 interface modulate activity (69). For example, Oprian and co-workers have shown that mutants of Lys296 result in constitutive activity (70) and that the magnitude of the activity is inversely correlated with residue size (71). They concluded that the electrostatic Lys296–Glu113 interaction and a steric contact involving the Lys296 side chain both contribute to preventing receptor activation, presumably by preventing motion of H7. A similar loss of steric interactions between Trp265 and Ala295 (adjacent to Lys296) occurs in the activation of the wild-type receptor. Specifically, as described above, motion of the retinal allows for the rotation of Trp265 away from Ala295 on H7, which in turn may facilitate a shift of H7 into an active orientation.

Conclusions—Our structural studies on the location of the retinal and surrounding residues in Meta II described above and elsewhere (49) suggest that the all-trans-retinal Schiff base is holding EL2 and the extracellular ends of the receptor in an active conformation. It is known that mutation of a number of residues that line the retinal binding site can significantly influence the light dependent activation of transducin (55, 72–75), emphasizing the intricate interplay between the all-trans geometry of the retinal and amino acids on the extracellular side of the receptor in stabilizing the active state.

Together these results provide a new working model for how structural changes in the retinal are involved in receptor activation and are coupled to structural changes in several functional microdomains described previously (18, 19). Retinal isomerization leads to steric strain within the retinal binding site between the β-ionone ring and H5, and between the C19/C20 methyl groups and EL2. These interactions trigger the displacement of EL2, deprotonation of the Schiff base nitrogen and protonation of Glu113. The retinal C19 and C20 methyl groups are involved in rearrangement of EL2, whereas the β-ionone ring leads to rearrangement of the hydrogen bonding network centered on H5. We propose that EL2 motion and SB deprotonation are coupled to the motion of H5 and are needed for the retinal and H7 to shift into their active positions. Motion of the β-ionone ring is also coupled to the motion of Trp265, which likely happens in concert with motion of the cytoplasmic ends of H6 and H7 and the rearrangement of the hydrogen-bonding network centered on the conserved NP.VXY sequence. Motion of helices H5, H6, and H7, in turn, is coupled to the rearrangement of electrostatic interactions involving the conserved ERY sequence at the cytoplasmic end of H3, exposing the G protein binding site on the cytoplasmic surface of the protein. The location of the retinal and reorganization of the protein upon receptor activation provide a structural basis for understanding the action of agonists and antagonists in the large family of class A GPCRs.

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