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Location of Trp265 in Metarhodopsin II: Implications for the Activation Mechanism of the Visual Receptor Rhodopsin

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Isomerization of the 11-*cis* retinal chromophore in the visual pigment rhodopsin is coupled to motion of transmembrane helix H6 and receptor activation. We present solid-state magic angle spinning NMR measurements of rhodopsin and the metarhodopsin II intermediate that support the proposal that interaction of Trp265$^{6.48}$ with the retinal chromophore is responsible for stabilizing an inactive conformation in the dark, and that motion of the β-ionone ring allows Trp265$^{6.48}$ and transmembrane helix H6 to adopt active conformations in the light. Two-dimensional dipolar-assisted rotational resonance NMR measurements are made between the C19 and C20-methyl groups of the retinal and uniformly ^{13}C-labeled Trp265$^{6.48}$. The retinal C20–Trp265$^{6.48}$ contact present in the dark-state of rhodopsin is lost in metarhodopsin II, and a new contact is formed with the C19 methyl group. We have previously shown that the retinal translates 4–5 Å toward H5 in metarhodopsin II. This motion, in conjunction with the Trp–C19 contact, implies that the Trp265$^{6.48}$ side-chain moves significantly upon rhodopsin activation. NMR measurements also show that a packing interaction in rhodopsin between Trp265$^{6.48}$ and Gly121$^{3.36}$ is lost in metarhodopsin II, consistent with H6 motion away from H3. However, a close contact between Gly120$^{3.35}$ on H3 and Met86$^{2.53}$ on H2 is observed in both rhodopsin and metarhodopsin II, suggesting that H3 does not change orientation significantly upon receptor activation.

Keywords: G protein-coupled receptor; solid-state NMR; magic angle spinning; retinal chromophore; rotamer toggle switch

Introduction

Rhodopsin is a member of the large family of G protein coupled receptors (GPCRs).[1] These receptors share a common architecture consisting of seven transmembrane (TM) helices. The conservation of amino acids and functional microdomains within the TM helices has led to the idea that there is a common mechanism for activation in the class A GPCRs.[2–4] Electron paramagnetic resonance (EPR) measurements[5,6] and cross-linking[6,7] of the cytoplasmic ends of the TM helices have shown that an essential element of the activation mechanism is an outward rotation of TM helix H6. The crystal structure of rhodopsin reveals that the retinal chromophore is buried within the seven TM helix bundle.[8,9] We have recently shown that the retinal translates toward H5 upon conversion to the active intermediate metarhodopsin II (meta II).[10] Retinal isomerization results in a large rotation of the C20 methyl group toward the second extracellular loop (EL2) rather than in a large displacement of the ionone ring toward H3 or H6.[10] On the basis of this trajectory, the major retinal–protein interactions that result from retinal isomerization and translation are between the β-ionone ring and Met207$^{5.42}$ on H5 and the retinal chain and

Abbreviations used: DARR, dipolar assisted rotational resonance; EL2, second extracellular loop; GPCR, G protein-coupled receptor; DM, *n*-dodecyl maltoside; Hx, transmembrane helix Hx; MAS, magic angle spinning; meta I, metarhodopsin I; meta II, metarhodopsin II; ROS, rod outer segment; TM, transmembrane.

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Trp265$^{6.48}$ on H6†. Solid-state NMR measurements of $^{13}C\cdots^{13}C$ internuclear distances can provide high-resolution constraints on these retinal–protein contacts in the activated receptor.[10] We have recently found that the interaction between Met207$^{5.42}$ and the β-ionone ring increases in the active meta II intermediate.[11] Here, we address how retinal–Trp265$^{6.48}$ contacts may be involved in motion of H6.

Trp265$^{6.48}$ is one of three conserved aromatic amino acid residues on H6 in the rhodopsin subfamily of the class A GPCRs. Trp265$^{6.48}$ lies in an arc formed by the 11-*cis* retinal chromophore and the side-chain of Lys296$^{7.43}$ (Figure 1). The aromatic side-chain of Trp265$^{6.48}$ has the lowest thermal factor in the rhodopsin crystal structure,[12] and is packed between Gly121$^{3.36}$ on H3 and Ala295$^{7.42}$ on H7. The indole nitrogen of Trp265$^{6.48}$ forms a water-mediated hydrogen bond to Asn302$^{7.49}$ of the highly conserved NPxxY sequence on H7. As a result of its location in the retinal binding site, Trp265$^{6.48}$ is well positioned to couple retinal isomerization to the motion of H6 and possibly H7. The current model for rhodopsin activation involves an outward rigid body rotation of H6.[6] Remarkably, in bacteriorhodopsin it has been shown that retinal isomerization is coupled to movement of Trp182 on transmembrane helix H6 and opening of the proton conduction pathway on the intracellular side of the retinal.[13] The aromatic tryptophan ring adjacent to the conjugated retinal chain has also been implicated in the primary photochemistry by inducing a large dipole in the retinal following light absorption.[14]

Trp265$^{6.48}$ is part of a well-conserved motif involving proline and aromatic amino acids. Pro267$^{6.50}$, with a sequence identity of 99%, is among the most highly conserved residues in the class A GPCRs. Computational and experimental studies suggest that Pro267$^{6.50}$ serves as a hinge to facilitate H6 motion.[15–18] Clustered around Pro267$^{6.50}$ are three conserved aromatic amino acid residues: Phe261$^{6.44}$ (85%), Trp265$^{6.48}$ (85%) and Tyr268$^{6.51}$ (70%). These amino acid residues (along with a conserved Phe at position 6.52) are generally recognized as important in both ligand-binding and receptor activation.[19]

Several models have been proposed for how the Pro-aromatic motif couples retinal isomerization or ligand binding to receptor activation. On the basis of photochemical crosslinking experiments, Borhan *et al.*[20] have proposed that the 11-*cis* retinal chromophore in the ground state of rhodopsin serves as an inverse agonist by constraining the conformation of Trp265$^{6.48}$ and Phe261$^{6.44}$ on H6. Retinal isomerization moves the ionone ring away from Trp265$^{6.48}$ and eliminates the contact between

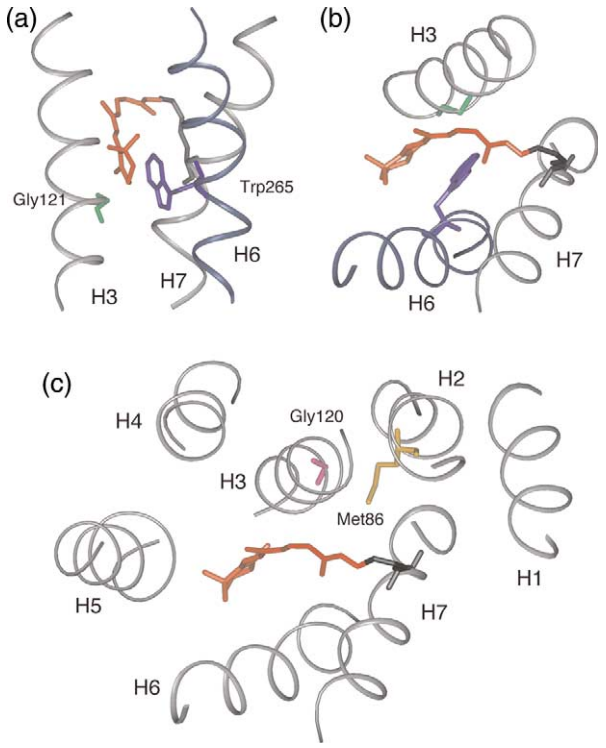

Figure 1. Structure of the retinal binding site of rhodopsin. (a) View of transmembrane helices H3, H6 and H7 in the crystal structure of rhodopsin. The retinal chromophore (red) is attached to Lys296 (black) on H7 and packs around Trp265$^{6.48}$ (blue) on H6. Trp265$^{6.48}$ packs against Gly121$^{3.36}$ (green) on H3. (b) View from the extracellular side of rhodopsin of H3, H6 and H7. (c) View of H1–H7 from the extracellular side of rhodopsin with the retinal (red), Lys296 (black), Met86$^{2.53}$ (orange) and Gly120$^{3.35}$ (purple) highlighted.

the C19 methyl group of the retinal and Phe261$^{6.44}$. This model contrasts with that proposed by Spooner *et al.*[21] in which the β-ionone ring does not move upon retinal isomerization and rhodopsin activation. Instead, deprotonation of the Schiff base following retinal isomerization leads to disruption of a hydrogen bonding network connected to Tyr268$^{6.51}$ on the extracellular side of H6. In this model, motion of the Tyr268$^{6.51}$ side-chain is associated with a "conformational switch" involving changes in the backbone ϕ and ψ torsion angles of Trp265$^{6.48}$ and Ala269$^{6.52}$, and outward rotation of the cytoplasmic segment of H6. In the ligand-activated class A GPCRs, Javitch and co-workers[18] proposed the "rotamer toggle switch" for receptor activation. In these receptors, ligand binding is thought to induce a change in the side-chain conformation of Phe$^{6.52}$, which is coupled to changes in the side-chain conformations of Cys264$^{6.47}$, Trp265$^{6.48}$ and Pro267$^{6.50}$.

Here, we use solid-state magic angle spinning (MAS) NMR spectroscopy to obtain structural constraints within the meta II intermediate between Trp265$^{6.48}$ and the retinal chromophore, and

† The nomenclature in superscript follows the convention established by Ballesteros & Weinstein[60] where the helix number is followed by the sequence position relative to the most conserved residue in the helix designated as 50.

between Met86$^{2.53}$ on H2 and Gly120$^{3.35}$ on H3. These new constraints provide insight into the role that Trp265$^{6.48}$ plays in stabilizing the inactive state of rhodopsin and in coupling retinal isomerization to helix motion upon photoactivation.

Results

Motion of transmembrane helix H6 relative to H3 in metarhodopsin II

The relative motion of TM helices 3 and 6 is crucial for rhodopsin activation. Using rhodopsin containing nitroxide spin labels at positions on the intracellular ends of the TM helices, Hubbell and Khorana found that H6 exhibits the largest displacement of the seven TM helices upon receptor activation.[6] Cross-linking studies using cysteine disulfide formation[6] and His-Zn coordination[7] have shown that it is possible to block receptor activation by restraining the motion of the intracellular ends of H3 and H6. It is less well established whether H3 itself moves significantly within the TM helix bundle upon receptor activation. Gly121$^{3.36}$ is strictly conserved in the opsin subfamily and provides a packing surface for Trp265$^{6.48}$. We have shown that mutation of Gly121$^{3.36}$ in the H3–H6 interface to larger amino acids leads to rhodopsin activity in the dark,[22,23] and have proposed that H3 and H6 must reorient relative to each other in order to accommodate the larger amino acid side-chains.[24] To directly confirm that the Gly121$^{3.36}$–Trp265$^{6.48}$ distance changes in meta II, we incorporated isotope labeled glycine and tryptophan into rhodopsin and used the DARR NMR ^{13}C–^{13}C recoupling experiment to determine their proximity.

Figure 2 presents two-dimensional (2D) DARR NMR spectra of rhodopsin containing U-^{13}C labeled tryptophan and $^{13}C^{\alpha}$-labeled glycine. The spectra were obtained in the detergent dodecylmaltoside (DM) in which the protein can adopt an active meta II conformation that appears to be identical to meta II formed in rod outer segment (ROS) membranes (see Materials and Methods). There are five tryptophan residues and 23 glycine residues in rhodopsin. In the dark state, the $C^{\zeta 3}$, $C^{\eta 2}$ and $C^{\varepsilon 2}$ carbon atoms of Trp265$^{6.48}$ are within ~5.5–6.0 Å of the C^{α} carbon of Gly121$^{3.36}$; this is the distance range of the DARR experiment. Figure 2 shows the region of the DARR NMR spectrum containing the tryptophan aromatic resonances in one dimension (110–140 ppm) and the α/β carbon resonances in the second dimension (20–60 ppm). Uniform ^{13}C labeling of tryptophan results in broad, intense cross-peaks between 20–35 ppm and 50–60 ppm. These peaks arise from the short intra-residue distances between the aromatic carbons and the C^{α} and C^{β} carbon atoms within each tryptophan. The C^{α} carbon resonance of glycine (40–45 ppm) is well resolved from all other amino acid C^{α} carbons.

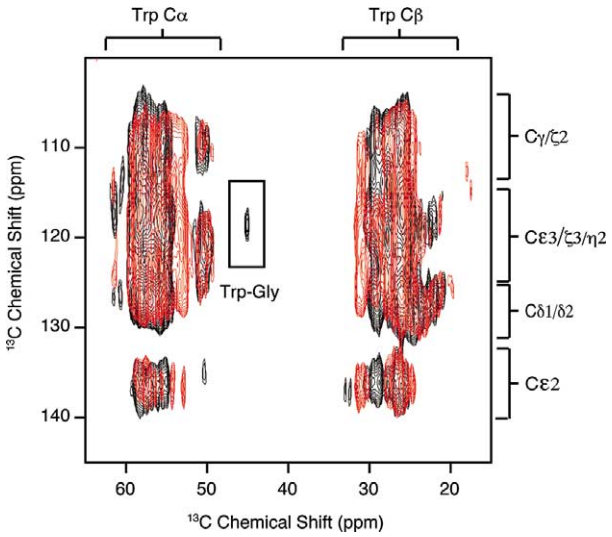

Figure 2. Tryptophan–glycine contacts in rhodopsin and meta II. ^{13}C 2D DARR NMR spectra of rhodopsin (black) and meta II (red) labeled with [U-^{13}C] tryptophan and [α-^{13}C]glycine. Only the region of the spectrum containing the cross-peaks between the tryptophan aromatic carbon atoms and the α and β carbon atoms is shown. In rhodopsin, a cross-peak between Gly121$^{3.36}$ and Trp265$^{6.48}$ is observed, consistent with the crystal structure. A tryptophan–glycine contact is not observed in meta II.

In rhodopsin, the single Trp-Gly cross-peak assigned to the Trp265$^{6.48}$–Gly121$^{3.36}$ contact is observed at a tryptophan chemical shift of 119 ppm and a glycine chemical shift of 44.9 ppm. The Gly121$^{3.36}$ chemical shift is consistent with α-helical secondary structure.[25] Unfortunately, due to lack of resolution in the tryptophan region of the spectrum the chemical shift at 119 ppm can be assigned to any of the three $C^{\zeta 3}$, $C^{\eta 2}$ or $C^{\varepsilon 2}$ carbon atoms of the tryptophan indole ring†.[26] Nevertheless, the data obtained using [U-^{13}C] Trp and singly ^{13}C-labeled Gly demonstrate that we are able to detect an interhelical contact without problems associated with dipolar truncation.[27]

Upon illumination, the intensity of the Trp-Gly cross-peak is lost, indicating that the Trp265$^{6.48}$–Gly121$^{3.36}$ internuclear distance increases, placing it outside of the detection range of the DARR experiment. The loss of the Trp265$^{6.48}$–Gly121$^{3.36}$ contact may be due to rigid body motion of transmembrane helices H3 or H6 and/or rotation of the Trp265$^{6.48}$ side-chain.

In order to address the relative motion of H3, we measured the contact between Met86$^{2.53}$ and Gly120$^{3.35}$ in rhodopsin and meta II (Figure 1(c)). Gly120$^{3.35}$ is adjacent to Gly121$^{3.36}$ on H3. Met86$^{2.53}$ is on H2 with its ε-CH_3 group oriented into a space between helices H3, H6 and H7 created by

† http://www.bmrb.wisc.edu/ref_info/statful.htm

Gly120$^{3.35}$ and Ala299$^{7.46}$.[9] This contact is sensitive to the relative positions of H2 and H3.

Figure 3 shows 1D rows taken from a 2D DARR NMR spectrum of rhodopsin containing glycine ^{13}C-labeled at the α-carbon and methionine ^{13}C-labeled at the methyl group. There are 23 glycine residues and 16 methionine residues in rhodopsin, but only Met86$^{2.53}$ and Gly120$^{3.35}$ have an internuclear ^{13}C···^{13}C separation of less than 5.5 Å. The crystal structure of rhodopsin shows that the ε-CH_3 group of Met86$^{2.53}$ is 4.2 Å from the α-carbon of Gly120$^{3.35}$. The black row in Figure 3 is taken through the glycine C^{α} diagonal resonance at 44.9 ppm. We observe a single cross-peak at 13.2 ppm corresponding to the ε-CH_3 to C^{α} contact between Met86$^{2.53}$ and Gly120$^{3.35}$, consistent with the crystal structure. Upon conversion to meta II, the chemical shift of the cross-peak changes, but the intensity remains constant (red spectrum). This shows that the distance between the two labels changes by less than ∼1.0 Å.[28]

The chemical shift of the Met86$^{2.53}$ ε-CH_3 group changes from 13.2 ppm to 14.6 ppm, while the chemical shift of the Gly120$^{3.35}$ α-carbon shifts from 44.9 ppm to 46.5 ppm. Although the glycine chemical shift is consistent with an increase in helical secondary structure, the region of H3 that contains Gly120$^{3.35}$ is already a well-defined helix. The change in the glycine and methionine chemical shifts may result from a change in interactions with Trp265$^{6.48}$ due to the change in position of the aromatic tryptophan side-chain (see below).

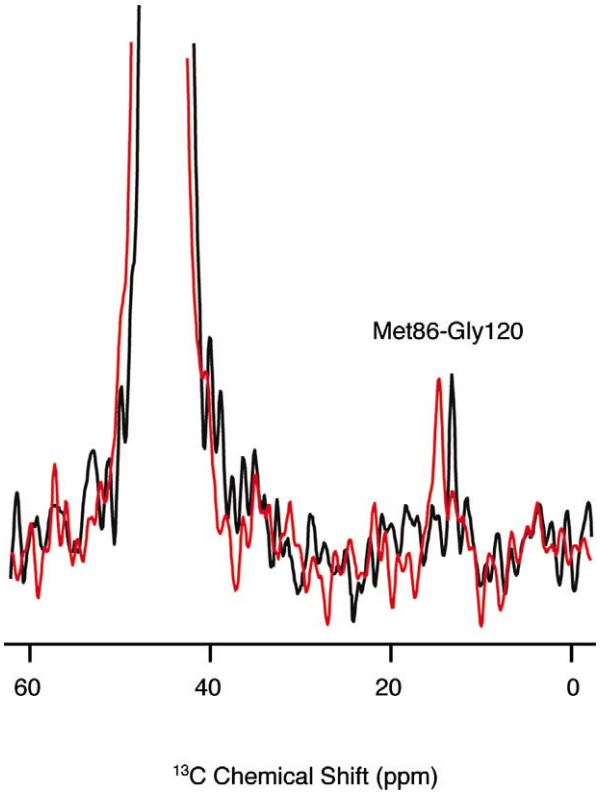

Figure 3. Methionine–glycine contacts in rhodopsin and meta II labeled with [ε-^{13}C]methionine and [α-^{13}C]glycine. Rows taken through the glycine diagonal at 44.9 ppm and 46.5 ppm of ^{13}C 2D DARR NMR spectra of rhodopsin (black) and meta II (red), respectively.

Tryptophan–retinal interactions in metarhodopsin II

The conservation of Trp265$^{6.48}$ in the opsin family of GPCRs and the conservation of aromatic amino acids at the extracellular end of H6 across the GPCR superfamily strongly suggest that Trp265$^{6.48}$ plays a functional role in rhodopsin activation. On the basis of retinal–protein contacts observed in meta II, we had previously argued that the translation of the retinal toward H5 was coupled to an outward rotation of H6.[10] Motion of the β-ionone ring toward H5 provides space for the Trp265$^{6.48}$ to reorient, and the highly conserved Pro267$^{6.50}$ provides flexibility for a global conformational change in H6 in response to or in concert with the motions of the retinal chromophore and the Trp265$^{6.48}$ side-chain. To determine the location of Trp265$^{6.48}$ relative to the retinal in meta II, we incorporated ^{13}C labels into tryptophan and the retinal chromophore for 2D DARR NMR experiments on rhodopsin and meta II.

Figure 4(a) presents the 2D DARR NMR spectrum of rhodopsin containing (U-^{13}C)-labeled tryptophan and regenerated with a retinal ^{13}C labeled at the C12 and C20 carbon atoms. The Figure shows the region of the spectrum that includes resonances of the tryptophan aromatic carbon atoms and the C12 carbon of retinal in one dimension (100–145 ppm) and the β carbon atoms and methyl groups of rhodopsin and the retinal in the second dimension (10–35 ppm). The large, unresolved resonances in both the rhodopsin and meta II spectra are from intra-residue correlations between the aromatic carbon atoms (105–140 ppm) and the C^{β} carbon (23–31 ppm) of tryptophan. The retinal C12–C20 cross-peak represents an internal control for the conversion to meta II. These labels are at a fixed separation in the retinal and the C12 chemical shift is sensitive to conformation of C11=C12 bond. The 131.8 ppm chemical shift observed for the C12 resonance in rhodopsin is similar to that reported.[29] Upon sample illumination, the C12 resonance shifts to 137.6 ppm, consistent with conversion to an all-*trans* retinal with an unprotonated Schiff base.[30] The change in the C20 chemical shift from 15.8 ppm to 13.2 ppm is also the same as that observed in our previous studies.[10] The cross-peak observed in rhodopsin (black spectrum, Figure 4(a)) between the C20 methyl group and the aromatic carbon atoms of tryptophan is consistent with the 3.9 Å Trp265$^{6.48}$–C20 distance in the crystal structure.[9] This cross-peak disappears upon activation (red spectrum), indicating that the side-chain of Trp265$^{6.48}$ is more than 5.5 Å from the C20 carbon of retinal in the active meta II intermediate.

Figure 4(b) presents the 2D DARR NMR spectrum of rhodopsin containing (U-^{13}C)-labeled tryptophan and regenerated with 11-*cis* retinal

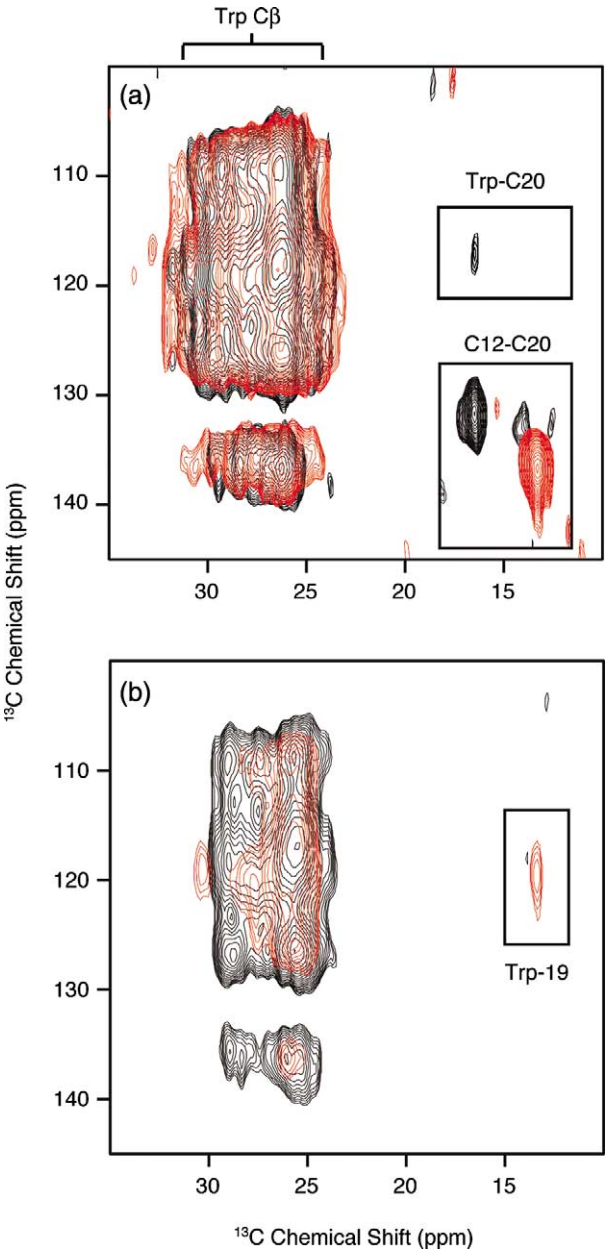

Figure 4. Tryptophan–retinal contacts in rhodopsin and meta II. (a) ^{13}C 2D DARR NMR spectra of rhodopsin (black) and meta II (red) labeled with [U-^{13}C]tryptophan and regenerated with retinal ^{13}C labeled at the C12 and C20 positions. A tryptophan-C20 cross-peak is observed in rhodopsin that is absent in meta II. The C12–C20 cross-peaks display the characteristic C12 and C20 chemical shifts of retinal in rhodopsin and meta II. (b) ^{13}C 2D DARR spectra of rhodopsin (black) and meta II (red) labeled with [U-^{13}C]tryptophan and regenerated with retinal ^{13}C labeled at the C15 and C19 positions. A tryptophan-C19 cross-peak is only observed in meta II.

^{13}C-labeled at the C15 and C19 carbon atoms. The same region is displayed as in Figure 4(a); the intense cross-peaks correspond to the directly bonded ^{13}C carbon atoms within the [U-^{13}C] Trp side-chain. The closest retinal C19–Trp265$^{6.48}$ distance in the crystal structure of rhodopsin is 6.1 Å,[9] which is at the upper limit of the DARR NMR experiment, and consequently no C19-Trp cross-peaks are observed. Upon conversion to meta II, the C19 methyl resonance shifts to 13.3 ppm,[10] and a cross-peak is observed with tryptophan at 120 ppm. The appearance of a cross-peak indicates that the aromatic side-chain of Trp265$^{6.48}$ is within 5 Å of the retinal C19 carbon. Trp265$^{6.48}$ is the only tryptophan within 12 Å of the retinal in the crystal structure. The C19 methyl group is likely in van der Waals contact with the Trp265$^{6.48}$ side-chain based on the stronger intensity of the C19-Trp cross-peak compared with the C20-Trp cross-peak in rhodopsin where the internuclear distance is 3.9 Å. The 120 ppm chemical shift is consistent with the closest tryptophan carbon to the C19 methyl group being either the $C^{\zeta 3}$, $C^{\varepsilon 3}$ or the $C^{\eta 2}$ carbon of the Trp indole ring†.[26]

Discussion

Evidence for a stable helical core in rhodopsin

Transmembrane helices H3 and H6 are critical for GPCR activation. The conserved ERY sequence is located at the cytoplasmic end of H3 and is involved in an ionic lock that keeps the receptor in an inactive orientation in the dark.[31–34] Cross-linking the intracellular ends of H3 and H6 blocks receptor activation.[6,7] Although the largest helix displacement is thought to occur in H6,[6] a "counterion switch" mechanism in rhodopsin activation has suggested a general model of GPCR activation that involves coupling of structural changes in EL2 to motion of H3.[35]

In contrast to the ideas presented above, we have proposed that H3 is part of a stable core of helices that does not change appreciably upon activation.[36] On the basis of an analysis of class A GPCR sequences, we found several positions where small (Gly, Ala) and weakly polar (Ser, Thr, Cys) amino acids are highly conserved when considered as a group with similar structural properties.[36] In membrane proteins these amino acids have high propensities for mediating close helix association.[37] We found that these group-conserved small and weakly polar amino acids in rhodopsin are located in helix interfaces, mainly involving helices H1 through H4, where they facilitate the formation of interhelical hydrogen bonds between amino acids with high sequence identities.[36] For instance, Ala82$^{2.49}$, Ala153$^{4.42}$, Ala164$^{4.53}$ and Ala168$^{4.57}$ mediate the close association of H2, H3 and H4 and permit an interhelical hydrogen bond between Asn78$^{2.45}$ and Trp161$^{4.50}$ that effectively locks these three helices together. The Asn78$^{2.45}$–Trp161$^{4.50}$ interaction does not appear to change in meta II.[38,39]

In this study, we have established an additional

† http://www.bmrb.wisc.edu/ref_info/statful.htm

contact within the H1–H4 bundle of helices that does not change upon rhodopsin activation. We observed that the cross-peak between Met86$^{2.53}$ on H2 and Gly120$^{3.35}$ on H3 has the same intensity in rhodopsin and meta II. These data support the model where H2, H3 and H4 do not move relative to one another in the region of the retinal upon receptor activation.[36]

Location of Trp265$^{6.48}$ in meta II

One of the main results in this study is that the new contact observed between Trp265$^{6.48}$ and the C19 methyl group in meta II allows us to distinguish between different models proposed for the role of Trp265$^{6.48}$ in rhodopsin activation. In the rotamer-toggle mechanism for GPCR activation, the rotamer states of Trp265$^{6.48}$ and a phenylalanine at position 6.52 are predicted to be coupled.[18] Stable structures are possible with a significant kink in the proline at position 6.50 and with the $\chi 1$ torsion angles of the amino acids at positions 6.48 and 6.52 in the $g+$ conformation. Phenylalanine at position 6.52 serves as the "ligand sensor", such that receptor agonists shift the $\chi 1$ torsion angle of Phe$^{6.52}$ to the *trans* conformation, while receptor antagonists are predicted to stabilize the $g+$ conformation.[40] The switch of the $\chi 1$ torsion angles of Trp$^{6.48}$ and Phe$^{6.52}$ to the *trans* conformation is associated with a straightening of H6 in the region of Pro$^{6.50}$.

The basic elements of the rotamer switch appear to be present in rhodopsin (Figure 5). Trp265$^{6.48}$ is in the $g+$ conformation with a significant kink at Pro267$^{6.50}$. Translation of the retinal chromophore toward H5 can facilitate a change of the $\chi 1$ torsion angle of Trp265$^{6.48}$ to *trans* by the removal of steric interactions with the β-ionone ring, i.e. motion of the β-ionone ring away from Trp265$^{6.48}$ removes a steric constraint that otherwise prevents the motion of the Trp side-chain.[10] In this case, the ionone ring in rhodopsin is serving the function of Phe$^{6.52}$ in the ligand-activated GPCRs by preventing the motion of Trp265$^{6.48}$ in the inactive receptor.[18]

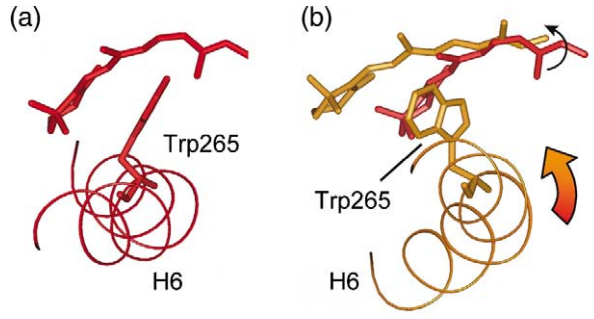

Figure 5. Model for structural changes in rhodopsin and metarhodopsin II. (a) A portion of the rhodopsin crystal structure showing the position of the 11-*cis* retinal relative to Trp265$^{6.48}$ and H6 viewed from the extracellular side of the receptor. (b) Structural model of meta II showing the positions of the all-*trans* retinal, Trp265$^{6.48}$ and H6 viewed from the extracellular side of the receptor. The position of the retinal in rhodopsin is shown in red.

The observation of the contact between the retinal C19 methyl group and Trp265$^{6.48}$ indicates that the indole side-chain moves into the space opened up by translation of the β-ionone ring toward H5. This mechanism is in agreement with the model proposed by Borhan *et al*.[20] in which the β-ionone ring moves away from Trp265$^{6.48}$. This mechanism differs from the model proposed by Spooner *et al*.[21] in which the β-ionone ring does not change position in meta II and Trp265$^{6.48}$ moves away from the C19 methyl group upon rhodopsin activation.

Several independent observations are consistent with a rotamer switch in rhodopsin. First, UV absorbance studies show that Trp265$^{6.48}$ moves into a more hydrophilic environment and that the plane of the indole side-chain of Trp265$^{6.48}$ changes from an orientation parallel to the bilayer normal to an orientation roughly perpendicular to the bilayer normal.[39,41] Second, motion of the side-chain of Trp265$^{6.48}$ away from H7 is consistent with weakening of the water-mediated hydrogen bond between the indole nitrogen of Trp265$^{6.48}$ and Asn302$^{7.49}$ on H7.[38,39]

The observed Trp265–C19 contact adds to the constraints previously obtained on the meta II structure from solid-state NMR.[10,38] One of the unexpected results of our previous studies was that the C20 methyl group moves to within ~5 Å of Tyr178, while the C19 methyl group moves out of the contact regions of all of the tyrosine residues in the retinal binding site. As a result, both the C19 and C20 methyl groups are oriented toward extracellular loop 2 (EL2) in meta II. The C19 methyl group is in close proximity to Trp265$^{6.48}$ and >5.5 Å from any tyrosine (4′-^{13}C), while the C20 methyl group is within 5.5 Å of Tyr178 (4′-^{13}C) and is no longer in contact with Trp265$^{6.48}$. The large motion of the Trp265$^{6.48}$ side-chain is presaged by a significant shift toward the extracellular surface of electron density attributed to Trp265$^{6.48}$ in the 5.5 Å meta I structure.[42]

The motion of Trp265$^{6.48}$ casts new light on the retinal binding site in rhodopsin and how the receptor is locked off in the dark. Trp265$^{6.48}$ is highly conserved in the visual pigments (91%). The side-chain of Trp265$^{6.48}$ has the lowest thermal factor in the crystal structure of rhodopsin and the largest contact area with the retinal.[12] Importantly, the side-chain is located in the arc created by the 11-*cis* retinal and Lys296. The tight packing interaction insures that the Trp265$^{6.48}$ side-chain will not rotate until the retinal isomerizes upon light absorption. In fact, binding of 11-*cis* retinal in the retinal binding site is known to eliminate the high basal activity of the opsin apoprotein, a feature that is critical for a photoreceptor.[43]

Trp265$^{6.48}$ is likely to be only one of several elements of the activation mechanism. Amino acid positions where mutation leads to constitutive activity are found on several of the TM helices. For instance, the ERY sequence at the cytoplasmic end of H3 is part of an "ionic lock" that constrains the position of H3 relative to H6 in the inactive

receptor.[8,33,34] Mutation of Glu134$^{3.49}$ to Gln leads to constitutive activity.[31,32] In addition, EL2 plays an important role in activation. Amino acids on EL2 contribute to a hydrogen-bonding network that is thought to maintain rhodopsin in an inactive conformation[44] and are critical to the counterion switch that has been proposed as part of the activation mechanism.[35] In the rhodopsin crystal structure, EL2 acts as a wedge, preventing the extracellular ends of H6 and H7 from moving in toward the core of the protein. The importance of EL2 is reflected in several other class A GPCRs. For instance, the melanocortin receptors are one of the few GPCR subgroups that are regulated by both agonists and inverse agonists.[45] The high degree of basal activity found in these receptors has been attributed to the lack of a large EL2. These observations have led Schwartz and colleagues to propose that H6 has an active inward orientation.[45] Due to the lack of a large EL2 in the melanocortin receptors, H6 and H7 may be free to move into the space that is occupied by EL2 in the rhodopsin structure, leading to constitutive activity. Experiments are in progress to establish whether EL2 changes position in rhodopsin to allow the extracellular end of H6 to move inward while the intracellular end tilts out[6] with Pro267$^{6.50}$ as the pivot point.

Materials and Methods

Expression and purification of ^{13}C-labeled rhodopsin

Rhodopsin was expressed in stable tetracycline-inducible HEK293S cells[46] containing the wild-type opsin gene.[47] Fetal bovine serum (FBS) was dialyzed three times against 20 l of buffer A (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.2).[48] The cells were grown in Dulbecco's modified Eagle's medium formulation[49] prepared from cell culture-tested components (Sigma, St. Louis, MO). The suspension growth medium was supplemented with specific ^{13}C-labeled amino acids (Cambridge Isotope Laboratories, Andover, MA), dialyzed fetal bovine serum (10%), Pluronic F-68 (0.1%), heparin (50 mg/l), penicillin (100 units/ml) and streptomycin (100 μg/ml).[48,50] The cells were induced on day 5 with 2 mg/l tetracycline[46] and harvested on day 7.

The HEK293S cell pellets were resuspended in buffer A (40 ml/l cell culture + protease inhibitors).[47] Unlabeled 11-*cis* retinal was added in two steps to a final concentration of 15 μM. The cells (40 ml/l of cell culture) were solubilized for 4 h at room temperature using buffer B (buffer A + 1% DM).

Samples were purified by affinity chromatography using the 1D4 antibody (National Cell Culture Center, Minneapolis, MN).[47] After loading the solubilized cell membranes onto the 1D4 antibody column, the column was washed with 25 column volumes of buffer C (buffer A + 0.05% DM), followed by equilibration with 25 column volumes of buffer D (2 mM NaH_2PO_4/Na_2HPO_4 (pH 6) + 0.02% DM). Buffer D containing the last nine C-terminal amino acid residues of rhodopsin as the antibody epitope was used for elution. Eluted samples were concentrated in Centricon cones (Amicon, Bedford, MA) with a 10 kDa cutoff to a volume of 1 ml.

Synthesis of ^{13}C-labeled retinals and regeneration into rhodopsin

Synthetic retinals with specific ^{13}C labels were produced by standard methods.[51] The 11-*cis* isomer was purified by isocratic high pressure liquid chromatography with a dried solution of 96% hexane, 4% ethyl acetate at 8 ml/min using an Alltech (Deerfield, IL) Econosphere 10 μl silica column.

The rhodopsin pigments in DM micelles were regenerated with ^{13}C-labeled retinals by illumination of concentrated samples in the presence of a 2:1 molar ratio of retinal-to-protein as discussed previously.[10] Regeneration was typically greater than 80% as determined by recovery of the 500 nm visible absorption band. The regenerated sample was then concentrated to a volume of 100 μl or less by water evaporation using a stream of argon gas. Samples were then transferred into a 4 mm MAS rotor and frozen at −80 °C until NMR data acquisition.

Trapping of the metarhodopsin II intermediate

Samples were illuminated in the NMR rotor using a 400 W lamp with a >495 nm cutoff filter for 20 s at room temperature and immediately put in the NMR probe. The sample was frozen within 3 min of illumination using N_2 gas cooled to −80 °C. The chemical shifts of the carbon atoms of the polyene chain of the retinal are sensitive to both protonation and isomerization state. To confirm that we have trapped meta II and completely converted the sample from rhodopsin and meta I, we monitor the chemical shift changes of the ^{13}C labels on the C12 and C15 carbon atoms of the retinal.

The meta II trapping experiments are carried out in detergent in order to convert the sample quantitatively to the meta II intermediate. We observe no residual rhodopsin or meta I after illumination, and typically are able to trap >85% of our original sample in the meta II state. Meta II is the only intermediate with an all-*trans* retinal and an unprotonated Schiff base. We do not have a similar way to assess whether the conformation of the protein in DM detergent is identical to that in ROS membranes. The two strongest arguments that there are (at a minimum) no significant differences are (1) meta II in DM is able to activate transducin[52,53] and (2) the observed vibrational frequencies in FTIR difference spectra of meta II minus rhodopsin are identical for rhodopsin in DM or ROS membranes.[54,55] As a result, the detergent-stabilized meta II structure captures the critically important conformational features of the activated receptor. Importantly, the meta II intermediate we have trapped is well-defined spectroscopically. The NMR resonances of both the retinal and protein are narrow, single peaks. We have not observed linebroadening or splittings characteristic of a disordered intermediate(s) or substates of the meta II (i.e. we do not observe two meta II species, such as meta IIa and meta IIb). The half mean time for proton uptake of meta II in DM is 25 ms.[56] As mentioned above the time between illumination and freezing of the sample is approximately 3 min. Therefore we can assume that the protonation uptake in our sample is complete and we are observing the form of meta II that activates transducin. Thus, we have trapped a well-defined intermediate between meta I and meta III containing a retinal

chromophore whose structure is characteristic of meta II and whose protein vibrations are the same as in meta II trapped in ROS membranes. The intermediate is functionally equivalent to meta II in ROS membranes as it can activate transducin.

NMR spectroscopy

The solid state NMR experiments were run on a 360 MHz or 600 MHz Bruker AVANCE spectrometer using 4 mm MAS probes. The MAS spinning rate for each sample was selected to avoid overlap of MAS sidebands with ^{13}C cross-peaks. Ramped amplitude cross-polarization[57] contact times were 2 ms in all experiments and two pulse phase modulated[58] decoupling was used during the evolution and acquisition periods. The decoupling field strength was typically 90 kHz. ^{13}C chemical shifts were referenced to external TMS. The samples were maintained at $-80\,^{\circ}C$ for the entire acquisition.

For DARR experiments,[59] mixing times of 600 ms were used to maximize homonuclear recoupling between ^{13}C labels.[28] The ^{1}H radiofrequency field strength during mixing was matched to the MAS speed for each sample, satisfying the $n=1$ matching condition. Each 2D data set represents between 1536 and 2560 scans in each of 64 rows in the $f1$ dimension. 40 Hz of exponential line broadening was used in the $f2$ dimension and a cosine multiplication was used in the $f1$ dimension along with a 32 coefficient forward linear prediction.

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