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Free backbone carbonyls mediate rhodopsin activation

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Abstract

Conserved prolines in the transmembrane helices of G protein-coupled receptors (GPCRs) are often considered to function as hinges that divide the helix into two segments capable of independent motion. Depending on their potential to hydrogen-bond, the free C=O groups associated with these prolines can facilitate conformational flexibility, conformational switching or stabilize receptor structure. To address the role of conserved prolines in family A GPCRs, we focus on bovine rhodopsin, a GPCR in the visual receptor subfamily, using solid-state NMR spectroscopy. The free backbone C=O groups on helices H5 and H7 are found to stabilize the inactive rhodopsin structure through hydrogen-bonds to residues on adjacent helices. In response to light-induced isomerization of the retinal chromophore, hydrogen-bonding interactions involving these C=O groups are released facilitating H5 and H7 repacking onto the transmembrane core of the receptor. These results provide insights into the multiple structural and functional roles prolines play in membrane proteins.

Prolines within the transmembrane (TM) helices of membrane proteins can function in several ways. First, they may facilitate protein dynamics as flexible hinges and play a functional role in guiding large scale conformational changes[1–3]. Second, prolines may be key elements in stabilizing protein structure. Proline-induced kinks can facilitate tighter packing of membrane proteins by allowing the helices to adopt optimal side chain interactions[4,5] and the backbone carbonyls at the *i-4* position relative to TM prolines are free to form strong stabilizing interhelical hydrogen bonds. Third, prolines and the associated free C=O groups can facilitate (reversible) switching between distinct protein conformations[6].

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Author Contributions

M.E., P.J.R and S.O.S conceived the study; N.K, A.P, and M.E. made samples; M.E. and M.Z. collected and analyzed NMR data; A.P. and O.B.S.R. analyzed the protein database for proline interactions; C.A.O constructed rhodopsin mutants; N.K., A.P., P.J.R. and S.O.S wrote the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

To address the role of prolines in the structure and function of GPCRs, we focus on the visual receptor rhodopsin. Rhodopsin serves as an on-off switch for light detection in the vertebrate retina[7]. Light energy absorbed by the retinal chromophore in rhodopsin drives the receptor from an inactive to an active conformation. The hallmark of the active state of rhodopsin (Metarhodopsin II or Meta II) is the outward rotation of the intracellular end of TM helix H6. Coupled with H6 motion are changes in the orientations of the adjacent helices H5 and H7. Helices H5, H6, and H7 each contains a proline residue in the middle of the TM sequence: Pro215$^{5.50}$, Pro267$^{6.50}$ and Pro303$^{7.50}$, respectively (residues are designated throughout the text according to the Ballesteros-Weinstein universal numbering system[8])(Fig. 1). These residues are the most conserved in each of these helices in the family A GPCRs. An additional proline (Pro291$^{7.38}$) with high sequence conservation within the visual receptor subfamily occurs at the extracellular end of H7.

The defining feature of a proline in a TM helix is that it is unable to form a backbone hydrogen bond to the carbonyl group one helical turn away. In the case of Pro215$^{5.50}$ and Pro303$^{7.50}$ on H5 and H7, respectively, the free i-4 backbone carbonyls form hydrogen-bonds with strongly polar residues on adjacent helices. However, the free backbone carbonyls associated with Pro267$^{6.50}$ and Pro291$^{7.38}$ are oriented toward the membrane lipids and do not hydrogen bond in the crystal structures of inactive rhodopsin[9,10], active opsin[11] or Meta II[12,13], suggesting instead that they allow the helical segments to easily swivel. Coordinated motion of the extracellular ends of H6 and H7 has been proposed as part of a general mechanism for GPCR activation[14,15] raising the possibility that the sequence stretching from Pro267$^{6.50}$ to Pro291$^{7.38}$, which includes extracellular loop (EL3), pivots upon activation.

Early studies on rhodopsin using FTIR spectroscopy indicated that conformational changes at one or more prolines occurred upon activation[16]. FTIR difference spectra revealed a large shift of the amide I vibration associated with a peptide bond adjacent to the amino-terminal side of a proline. The authors left open the possibility that the observed shift was due to *cis-trans* proline isomerization. More recent studies using non-native amino acid substitutions at conserved prolines in the D2 dopamine receptor ruled out the idea that *cis-trans* proline isomerization occurs, at least in this specific GPCR[17]. They found that the main function of proline was to introduce a break in the helix by removing a backbone NH. Introducing either cyclic, R-hydroxy, or N-methyl residues unable to form NH hydrogen bonds at the position of proline resulted in receptors with wild-type function. Consistent with this role, in several GPCRs mutation of proline to residues that are able to form backbone hydrogen bonds disrupts expression[18] and/or function[18,19].

Solid-state NMR spectroscopy in combination with isotope labeling of rhodopsin can be used to clarify the role of specific prolines by targeting the carbonyl groups that are four amino acids upstream of conserved prolines. These C=O groups are free to form alternative hydrogen bonds due to the lack of an NH hydrogen bond partner along their own helix backbone. Our focus is on the highly conserved *i-4* free C=O groups on helices H5, H6 and H7 whose ^{13}C chemical shifts are sensitive to both secondary structure and hydrogen bonding. Each of these free carbonyls is in a functionally important region of the protein (Supplementary Note 1). The His211$^{5.46}$ carbonyl associated with conserved Pro215$^{5.50}$ on

H5 is located within the retinal-binding site, and is a key determinant of the high sensitivity of the dim light photoreceptors in rod cells, as compared to the color photoreceptors in cone cells[20]. The Ile263$^{6.46}$ carbonyl associated with conserved Pro267$^{6.50}$ is bracketed by conserved residues (Phe261$^{6.44}$ and Trp265$^{6.48}$) on TM helix H6 that are part of a transmission switch that couples retinal isomerization to conformational changes on the intracellular side of the receptor[21,22]. Finally, the Ala299$^{7.46}$ carbonyl associated with conserved Pro303$^{7.50}$ serves to orient the side chain of Asn55$^{1.50}$, the most conserved residue within the family A GPCRs[7]. The ^{13}C chemical shift changes of these free carbonyls upon receptor activation reveal that they play both structural roles in guiding how the TM helices pack in the inactive receptor, as well as functional roles in allowing the helices to reassemble in response to light-induced isomerization of the retinal chromophore. These results highlight the importance of hydrogen-bonding interactions involving the free C=O groups associated with TM prolines in membrane proteins.

RESULTS

The TM core of rhodopsin is highly conserved and serves to couple structural changes on the extracellular side of the receptor containing the retinal chromophore to the intracellular G protein binding region (Fig. 1). Crystal structures of the inactive[9,10] and active[11–13] states of rhodopsin reveal the positions of the conserved prolines and their associated free C=O groups relative to surrounding amino acids and structural water. These structures provide an essential framework for understanding how changes in hydrogen bonding guide the rearrangement of TM helices H5-H7. NMR spectroscopy was used here to target the free carbonyl groups associated with the conserved prolines within the TM core of rhodopsin. NMR measurements of specific ^{13}C=O chemical shifts report on the strength of the hydrogen bonding interactions in the inactive state and how they change upon activation with and without the Gα peptide.

Pro215$^{5.50}$

The conserved proline on helix H5 at position 215 in rhodopsin frees the carbonyl of His211$^{5.46}$. There are six histidine residues in rhodopsin that must be considered. The magic angle spinning (MAS) NMR difference spectrum between the inactive (dark) state of rhodopsin and the active Meta II intermediate showed that at least one His ^{13}C=O resonance shifts to lower frequency upon activation (Fig. 2a). The ^{13}C=O resonance that changes chemical shift can be assigned to His211$^{5.46}$ by using rotational echo double resonance (REDOR)[23] NMR filtering of rhodopsin specifically labeled with 1-^{13}C histidine and ^{15}N-phenylalanine. This labeling strategy generates a single ^{13}C-^{15}N labeled peptide bond as there is only a single His-Phe pair in the rhodopsin sequence, namely His211$^{5.46}$-Phe212$^{5.47}$. The REDOR experiment allows one to measure ^{13}C…^{15}N dipolar couplings in the solid-state NMR experiments, and the strong dipolar coupling resulting from the directly bonded ^{13}C-^{15}N pair allows one to selectively observe only the single ^{13}C=O resonance in the REDOR filtered spectrum[23]. Using the REDOR filtering experiment, we observed the ^{13}C=O frequency of H211$^{5.46}$ at 172.5 ppm in rhodopsin and at 170.2 ppm upon conversion to Meta II (Fig. 2a).

The decrease in the His211$^{5.46}$ ^{13}C=O chemical shift in the transition to Meta II can result from changes in hydrogen bonding, backbone torsion angles or both. Table 1 lists the ^{13}C chemical shifts of the free C=O groups on H5, H6 and H7 along with the backbone torsion angles associated with these residues in inactive and active structures. The chemical shift changes associated with changes in backbone conformation versus hydrogen bonding can be comparable in magnitude (4–5 ppm)[24–26]. The X-ray crystal structure of rhodopsin shows that the C=O of His211$^{5.46}$ is directly hydrogen-bonded to the Glu122$^{3.37}$ COOH side chain[9,10]. Nevertheless, the observed chemical shift of 172.5 ppm is lower than that normally observed for hydrogen-bonded C=O groups in α-helices suggesting that the lower chemical shift is caused by non-helical backbone torsion angles (bold, italics in Table 1). Indeed in the crystal structure of rhodopsin, the ϕ and ψ torsion angles show a strong distortion compared to standard α-helices (ϕ= −60° and ψ = −45°). Upon activation, the His211$^{5.46}$ C=O hydrogen bond with Glu122$^{3.37}$ is broken and a new hydrogen bond is formed between Glu122$^{3.37}$ and the His211$^{5.46}$ imidazole nitrogen[11,27] (see Fig. 2b). The direct His211$^{5.46}$ - Glu122$^{3.37}$ interaction can already be sensed using FTIR spectroscopy in the Meta I intermediate[28]. In the Meta II crystal structure[12,13], the helix is less distorted than in rhodopsin (the backbone torsion angles of His211$^{5.46}$ are closer to standard α-helix values), which would favor a downfield shift in the His211$^{5.46}$ ^{13}C=O resonance. The observed upfield chemical shift of the His211$^{5.46}$ C=O in Meta II is in the opposite direction and consequently is attributed to the loss of Glu122$^{3.37}$ hydrogen bonding. This change suggests that the His211$^{5.46}$ C=O functions as a hydrogen-bonding switch in receptor activation.

Pro267$^{6.50}$

Pro267$^{6.50}$, the conserved proline in TM helix H6, is associated with the free carbonyl of Ile263$^{6.46}$. There are 22 isoleucine residues in rhodopsin. In contrast to the histidine C=O difference spectrum (Fig. 2a), the MAS NMR difference between rhodopsin and Meta II containing 1-^{13}C isoleucine showed that several isoleucines change chemical shift upon activation (Fig. 2c). We took advantage of the unique Ile263$^{6.46}$-Cys264 pair to identify the Ile263$^{6.46}$ resonance in this spectrum. In the REDOR NMR filtered spectrum of rhodopsin (middle panel) labeled with 1-^{13}C-isoleucine and ^{15}N-cysteine, we observed a single resonance at 171.9 ppm assigned to Ile263$^{6.46}$. The Ile263$^{6.46}$ C=O group is oriented away from the helical bundle (Fig. 2d), toward the lipids, and the 171.9 ppm chemical shift likely reflects the absence of a hydrogen-bonding partner since the backbone ϕ and ψ torsion angles of Ile263$^{6.46}$ are close to values observed in standard α-helices.

Upon activation, we found that the Ile263$^{6.46}$ resonance split into two components at 170.5 and 175.6 ppm. In the Meta II crystal structure[12,13], the backbone ϕ and ψ torsion angles are still close to values in standard α-helices (Table 1). The upfield (170.5 ppm) component of the Ile263$^{6.46}$ ^{13}C=O resonance is consistent with a non-hydrogen bonded C=O and the small (1.4 ppm) upfield chemical shift would be consistent with a small distortion of the backbone from standard ϕ and ψ torsion angles. In contrast, the most likely explanation for the resonance with the large downfield chemical shift is that the Ile263$^{6.46}$ C=O establishes a strong hydrogen bonding interaction upon activation. The crystal structures of Meta II[12,13] reveal that the only side chain near the Ile263$^{6.46}$ C=O is the aromatic ring of Phe294$^{7.41}$.

Pro303$^{7.50}$

Conserved Pro303$^{7.50}$ on helix H7 frees the carbonyl of Ala299$^{7.46}$ in rhodopsin. With the HEK293S expression system used for ^{13}C-labeling rhodopsin, alanine is scrambled and cannot be specifically labeled. To target this carbonyl, we mutated Ala299$^{7.46}$ to serine. The A299S rhodopsin mutant exhibits a 500 nm absorption band, and the photobleaching behavior and stability of Meta II are similar to the wild-type pigment (data not shown). The new Ser299$^{7.46}$ - Val300$^{7.47}$ dipeptide sequence is unique. Using the REDOR filtering experiment on 1-^{13}C Ser, ^{15}N-Val-labeled rhodopsin, we observed the Ser299 ^{13}C=O resonance at 173.1 ppm in rhodopsin and at 168.9 ppm in Meta II (Fig. 2e).

The Ala299$^{7.46}$ C=O group is hydrogen bonded to the side chain NH_2 of Asn55$^{1.50}$ (Fig. 2f). Like His211$^{5.46}$, the backbone ϕ and ψ torsion angles of Ala299$^{7.46}$ in rhodopsin are different from those in standard α-helices (Table 1), but in Meta II the values are consistent with α-helix. The large decrease in chemical shift of the Ser299 ^{13}C=O resonance upon activation is therefore attributed to a loss of hydrogen bonding to Asn55$^{1.50}$ (indicated by bold highlight in Table 1). Given the high conservation of both Asn55$^{1.50}$ and the free C=O group at position 299 across the family A GPCRs, this structural change upon activation is likely related to receptor function.

Pro291$^{7.38}$

Pro291$^{7.38}$ at the extracellular end of H7 is highly conserved (82%) in the visual receptor subfamily, and frees the carbonyl at Phe287$^{7.34}$. There are two Phe-Met pairs, and correspondingly two peaks in the REDOR spectrum of rhodopsin at 174.1 ppm and 169.3 ppm (Fig. 2g). We assigned the 174.1 ppm to Phe85 in helix H2. This chemical shift is consistent with the helical secondary structure and backbone hydrogen-bonding of Phe85 in the rhodopsin crystal structure. The 169.3 ppm resonance is assigned to the Phe287$^{7.34}$ C=O on the basis of the ϕ and ψ torsion angles and the lack of a clear hydrogen bonding interaction. The Phe287$^{7.34}$ C=O is oriented toward the surrounding lipid and does not form inter-residue hydrogen bonds in the rhodopsin crystal structure[9,10] (Fig. 2h). Upon activation, we observed that the 174.1 ppm resonance splits into two components at 174 and 175.3 ppm and the resonance at 169 ppm broadens. We attribute the broadening (and lower intensity) to increased disorder in the backbone structure at Phe287$^{7.34}$, and unlike the other conserved prolines discussed above, this result is consistent with Pro291$^{7.38}$ functioning as a flexible hinge. The splitting of the 174.1 ppm resonance is tentatively assigned to conformational changes in the region of Gly89$^{2.56}$-Gly90$^{2.57}$, one helical turn from Phe85$^{2.52}$ in the rhodopsin sequence (see Discussion).

Induced fit of the C-terminal Gα peptide influences H6

The change in the ^{13}C=O resonance of Ile263$^{6.46}$ upon light activation is the most unusual of the three sites associated with the conserved prolines described above. A component of this ^{13}C=O resonance shifts downfield in frequency, but is not associated with a clear hydrogen-bonding partner in the crystal structures of active opsin[11] or Meta II[12,13]. Comparison of the crystal structures of rhodopsin and Meta II shows that the backbone torsion angles of Ile263$^{6.46}$ do not change appreciably upon activation. To better understand the structural changes occurring in this region of H6, we have obtained REDOR NMR

spectra of the free carbonyls at His211$^{5.46}$, Ile263$^{6.46}$ and Ser299$^{7.46}$ in the presence of the transducin Gα peptide.

It is known that binding of the transducin Gα peptide to Meta II stabilizes the active structure[29]. The C=O chemical shifts of His211$^{5.46}$, Ile263$^{6.46}$ and Ser299$^{7.46}$ do not move appreciably upon Gα peptide binding (Figs. 3a–c). However, there is a marked increase in the intensity of the Ile263$^{6.46}$ C=O with the unusual downfield chemical shift. The change in populations suggests there is an induced fit of the Gα peptide into its intracellular binding site, which is allosterically coupled to the region containing the Ile263$^{6.46}$ C=O.

DISCUSSION

Prolines are unique in lacking a backbone NH, which effectively eliminates the ability of the carbonyl group at the *i-4* position to form an α-helical hydrogen bond. The three prolines in rhodopsin on helices H5, H6 and H7 are among the most conserved residues in the family A GPCRs, and consequently the free C=O groups at the *i-4* positions associated with these prolines are highly conserved as well. Since breaking a backbone hydrogen bond in a hydrophobic environment is estimated to cost ~4–5 kcal/mol of energy[30], the free *i-4* carbonyl group represents an energetically favorable site for hydrogen bond formation. Depending on their ability to hydrogen bond, the free C=O groups can stabilize protein structure (strong hydrogen bonds) or facilitate conformational dynamics (weak or no hydrogen bonds). Conformational switching of hydrogen-bonding interactions may substantially lower the energetic barrier for breaking interhelical contacts and as such mediate the conformational changes that underlie protein function[6]. Our studies focus on the hydrogen bonding interactions of the conserved free C=O groups on H5, H6 and H7 in rhodopsin to address their role in receptor structure and function. These prolines and their associated free C=O groups exhibit several different hydrogen-bonding interactions and consequently provide insights into the possible roles of prolines in membrane proteins.

The structure of rhodopsin reflects the evolutionary requirements of night vision, namely a photoreceptor finely tuned to balance low dark noise (via a stable structure) and high sensitivity (through rapid receptor dynamics upon single photon absorption). The first four TM helices provide the stable framework onto which helices H5, H6 and H7 pack in the dark-state structure[7]. Helices H5, H6, and H7 each contain highly conserved prolines (Pro215$^{5.50}$, Pro267$^{6.50}$ and Pro303$^{7.50}$) and undergo the largest structural changes upon activation (Fig. 4a,b). The free C=O groups associated with these prolines are all roughly in the same plane within or near the receptor's TM core (Fig. 1). The TM core is highly conserved and involved in both receptor structure and function[7].

Structural roles likely exist for all three highly conserved prolines. As the receptor folds during protein synthesis, the free carbonyls at His211$^{5.46}$ and Ala299$^{7.46}$ in the middle of the TM helices are in a position to hydrogen bond to the strongly polar Glu122$^{3.37}$ and Asn55$^{1.50}$ side chains on helices H3 and H1, respectively. Computational studies on receptor stability and unfolding highlight His211$^{5.46}$ and Ala299$^{7.46}$ as the major TM residues that contribute to the overall structural integrity of rhodopsin[31]. These studies, which assessed receptor stability after removing *single* TM hydrogen bonds, provide strong support for the

role of the free C=O groups on H5 and H7 in stabilizing receptor structure. Although His211$^{5.46}$ and Ala299$^{7.46}$ were identified using two slightly different computational approaches, both results are consistent with His211$^{5.46}$ and Ala299$^{7.46}$ being sites of autosomal dominant retinitis pigmentosa (ADRP) mutations, which are typically associated with protein misfolding [32]. Mutation of Pro267$^{6.50}$ also leads to protein misfolding and ADRP. Since the free C=O associated with Pro267$^{6.50}$ does not hydrogen bond in inactive rhodopsin, its structural role may be to allow the helical segments on each side of Pro267$^{6.50}$ to optimize contacts with the other TM helices and possibly prevent non-native (misfolded) interactions[33].

Functional roles of the three highly conserved prolines likely involve conformational switching rather than conformational dynamics. Upon activation, both the His211$^{5.46}$ and Ser299$^{7.46}$ C=O groups lose their stabilizing hydrogen bonding interactions, which appear to act as molecular linchpins in rhodopsin. That is, they are stabilizing when in place, but result in a rearrangement of interhelical interactions and helix orientations when broken (Fig. 4c,d). The balance between the active and inactive conformations is dependent on whether the retinal-binding site is occupied by an 11-*cis* retinal PSB (inverse agonist) or all-*trans* retinal SB (agonist) chromophore. For H5, the new helix orientation is stabilized not by an alternative hydrogen bonding partner for the His211$^{5.46}$ C=O, but instead by the formation of two new interhelical hydrogen bonds, one involving the His211$^{5.46}$ *side chain* with Glu122$^{3.37}$, and the other involving the interaction of Tyr223$^{5.58}$ with Arg135$^{3.50}$. Conservative mutations of either Glu122$^{3.37}$ or Tyr223$^{5.58}$ decrease the stability of Meta II[20,34], i.e. the active H5 conformation reverts back to an inactive conformation more readily. For H7, the loss of the hydrogen bonding interaction with Asn55$^{1.50}$ allows H7 to become more helical. Mutations that favor a helical (non-distorted) conformation, as in the P303A mutant, lead to hyperactivity of the receptor[35]. The presence of molecular switches involving free C=O groups may also exist at other positions that have high subfamily conservation in GPCRs. For example, a recent NMR study suggested that the free C=O associated with conserved Pro4.60 in the β_1-adrenergic receptor is part of a complex hydrogen-bonding network linking H4 and H5 that is modulated by ligand binding[36].

The observation of two distinct chemical shifts for Ile263$^{6.46}$ ^{13}C=O resonance suggests that Pro267$^{6.50}$ provides a hinge in H6, but that it is not conformationally dynamic. The ^{13}C=O resonances are sharp, rather than broadened as observed for Phe287$^{7.34}$. Consistent with this idea, position 6.46 is 80% conserved as Ile, Val or Leu and only 2% conserved as glycine, which is thought to facilitate dynamics when located at the *i-4* position relative to TM prolines[2]. The mixture of chemical shift changes of the Ile263$^{6.46}$ ^{13}C=O resonance indicates that H6 does not toggle to a fully active conformation until binding of the Gα peptide. In contrast to the C=O groups on H5 and H7, one component of the Ile263$^{6.46}$ ^{13}C=O resonance shifts downfield consistent with an increase in hydrogen bonding. Furthermore, the chemical shifts or intensities of the free C=O groups associated with H5 and H7 do not markedly change with the addition of the Gα peptide, suggesting that H5 and H7 have adopted their fully active conformations prior to G protein binding.

Of the four prolines studied, only Pro291$^{7.38}$ appears to be conformationally dynamic in the active state. The broadened NMR resonances indicate that there is not a distinct

conformation in this region of the active receptor. For Pro215$^{5.50}$ and Pro303$^{7.50}$ on H5 and H7, the identity of the side chain at the *i-4* position may contribute to the hinging motion of these helices. Glycine and proline are two residue types that may confer flexibility when present at the *i-4* position relative to proline[2]. Glycine occurs most often at position 5.46 (17%) in the family A GPCRs, with serine being the next highest conserved at this position (11%). Olfactory receptors are excluded from this analysis since position 5.50 is only moderately conserved as a proline (38%) in the olfactory receptor subfamily. For position 7.46, serine has the highest conservation (56%) in the family A GPCRs excluding the olfactory receptors. Interestingly, both positions 7.46 (94%) and 7.50 (98%) are highly conserved as proline in the olfactory receptors. The presence of glycine or other small residues at position 5.46 in some class A GPCRs and proline at position 7.46 in the olfactory receptors may allow increased flexibility in H5 and H7 of these receptors, respectively.

Together, the NMR data presented here provide deeper insight into the diverse roles that proline can perform in rhodopsin. Key to understanding the functions of these prolines are the possible hydrogen bonding interactions involving the free *i-4* carbonyls. The free i-4 carbonyls can form stabilizing interactions in rhodopsin and function as conformational switches in the conversion to the active Meta II intermediate. As such, these findings are of broad significance in the structure and function of membrane proteins in general. In a survey of high-resolution membrane protein structures from the Protein Data Bank, we have found that ~75% of the free carbonyls have a polar residue nearby in a position to directly hydrogen bond or hydrogen bond via a water molecule (Supplementary Note 2). Many of these prolines occur in regions that are important in protein function, and they appear in membrane proteins as diverse as transporters, enzymes, and ion channels (Supplementary Note 2).

ONLINE METHODS

Expression and purification of ^{13}C labeled rhodopsin

Isotope enriched bovine opsin was expressed using inducible HEK293S cell lines. The original cell lines were obtained from Jeremy Nathans (Johns Hopkins University), but not authenticated or tested for mycoplasma contamination. HEK293S cells are widely used for production of recombinant proteins and viruses. The expressed opsin was generated into rhodopsin through incubation with ~30 micromolar 11-cis retinal, extracted from membranes using 1% (w/v) n-β-D dodecyl maltopyranoside (DDM) in PBS pH 7.4, and purified using Rho-1D4-Sepharose resin[37]. ^{13}C labeled retinal was prepared synthetically. Resin-bound rhodopsin was washed with 50 column volumes of 0.02% DDM in PBS pH 7.2, equilibrated with 0.02% DDM, 2 mM sodium phosphate pH 6.0. Rhodopsin was eluted in 0.02% DDM, sodium phosphate pH 6.0, 100 μM 9-mer elution peptide (TETSQVAPA) and concentrated to a volume of 1 ml using centricon (Millipore) centrifugation devices with a molecular weight cutoff of 30 kDa. The volume was further reduced to < 60 μl under a gentle stream of argon gas and packed into a 4 mm MAS rotor.

Solid-state NMR spectroscopy

Solid-state NMR experiments were conducted at static field strengths of either 500 or 600 MHz using a three-channel 4 mm MAS probe with a spinning rate of 10 kHz. Spectra were collected using a 2 ms contact pulse during cross polarization. SPINAL64 decoupling was used during acquisition with a ^{1}H RF field strength between 70–90 kHz. REDOR spectra were obtained with a dephasing period of 20 rotor cycles at 10 KHz MAS rate (2 ms)[38]. The REDOR filtered spectra (ΔS) were obtained by subtracting spectra with (S) and without (S_0) rotor-synchronized ^{15}N π pulses (10–11 μs). To reduce artifacts, S and S_0 spectral acquisition was interleaved and difference spectra were acquired scan-by-scan. ΔS spectra were summed over 60–100K scans using ~5–6 mgs of rhodopsin in a typical sample.

For rhodopsin and Meta II spectra, states were cryo-trapped at 190 K as described previously[39]. The spectra shown were generally repeated at least twice on samples purified from different cell growths without deviations of more than ±0.2 ppm in the reported chemical shifts. The ^{13}C high-resolution NMR and solid-state MAS NMR spectra were externally referenced to the ^{13}C resonance of neat TMS at 0 ppm at room temperature. Using TMS as the external reference, we calibrated the carbonyl resonance of solid glycine at 176.46 ppm. The chemical shift difference between ^{13}C of DSS in D_2O relative to neat TMS is 2.01 ppm.

Gα peptide synthesis and reconstitution with rhodopsin

The 15-mer C-terminal peptide of the Gα subunit of transducin (TDIIIKENLKDCGLF) was synthesized using solid-phase methods (Keck Small Scale Peptide Synthesis Facility, Yale University) and purified by reverse phase HPLC. The experiments on rhodopsin with added Gα peptide were carried in mixed micelles using DDM and dioleoylphosphoserine (DOPS). Solubilization of rhodopsin in DDM/DOPS mixed micelles has been shown to facilitate the interaction between rhodopsin and the full heterotrimeric form of transducin[40]. DOPS was added to rhodopsin in DDM micelles in a 1:100 rhodopsin to lipid ratio, and the Gα peptide to rhodopsin ratio was 8:1. The stability of the Meta II state was monitored by fluorescence quenching interaction between the indole of Trp265 and the β-ionone ring of the retinal chromophore[41].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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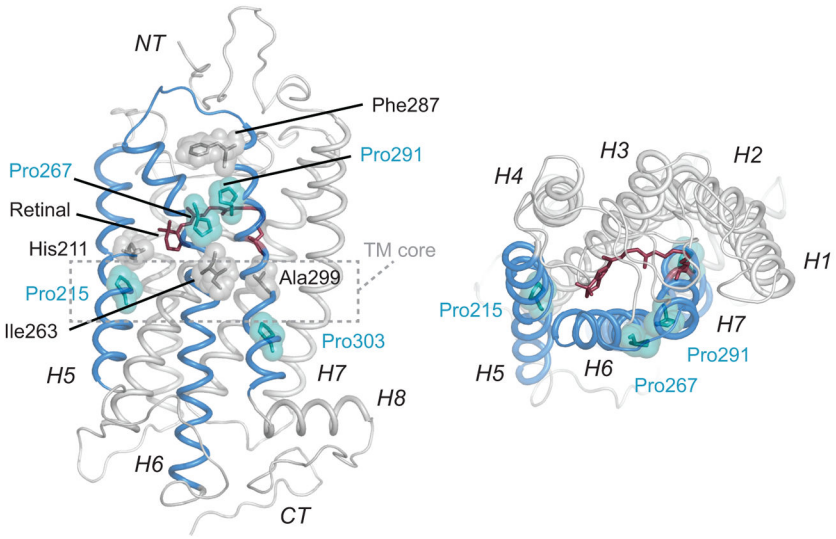

Figure 1.

Crystal structure of the visual receptor rhodopsin (PDB ID 1U19 [9]) showing the positions of Pro215$^{5.50}$, Pro267$^{6.50}$, Pro291$^{7.38}$ and Pro303$^{7.50}$. These prolines are located on helices H5, H6 and H7. The lack of an NH group results in a carbonyl group at the i-4 position from these prolines that is free to form interhelical hydrogen-bonds. The residues with the free carbonyl (His211$^{5.46}$, Ile263$^{6.46}$, Phe287$^{7.34}$, and Ala299$^{7.46}$) are shown in grey. The three free C=O groups that are conserved across the family A GPCRs (His211$^{5.46}$, Ile263$^{6.46}$, and Ala299$^{7.46}$) lie within or near the TM core of the receptor.

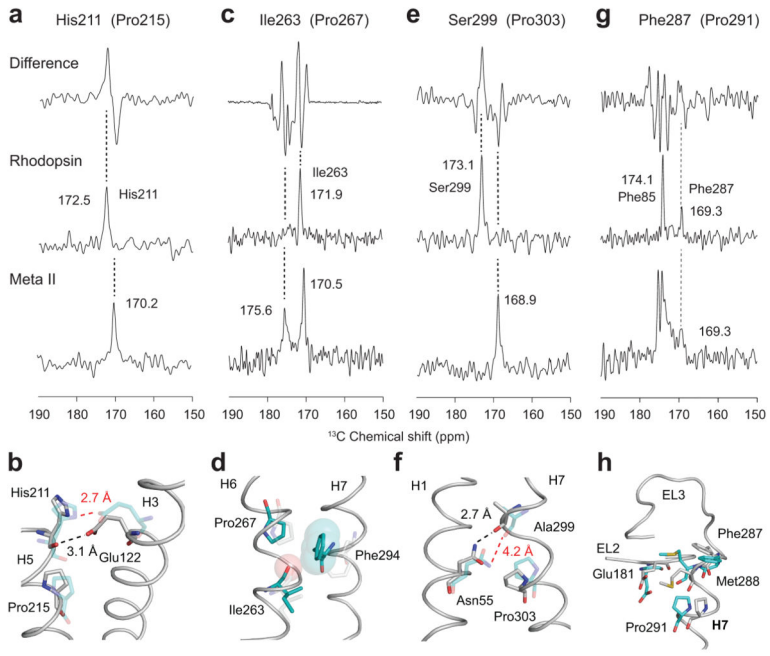

Figure 2.

REDOR NMR as a probe of hydrogen bonding changes of carbonyl residues at the i-4 positions of Pro215$^{5.50}$, Pro267$^{6.50}$, Pro291$^{7.38}$ and Pro303$^{7.50}$. (**a, c, e, g**) One-dimensional MAS NMR difference spectra between rhodopsin and Meta II are shown in the top panel. REDOR filtered spectra of rhodopsin and Meta II are shown in the middle and lower panels, respectively. The spectra highlight the i-4 for carbonyls associated with His211$^{5.46}$ using rhodopsin labeled with 1-^{13}C His, ^{15}N Phe (**a**), Ile263$^{6.46}$ using rhodopsin labeled with 1-^{13}C Ile, ^{15}N Cys (**c**), Ser299$^{7.46}$ using rhodopsin labeled with 1-^{13}C Ser, ^{15}N Val (**e**), and Phe287$^{7.34}$ using rhodopsin labeled with 1-^{13}C Phe, ^{15}N Met (**g**). (**b, d, f, h**) Crystal structures of rhodopsin (gray, PDB ID 1U19[9]) and Meta II (cyan, PDB ID 3PQR[13]) are shown in the region of interest.

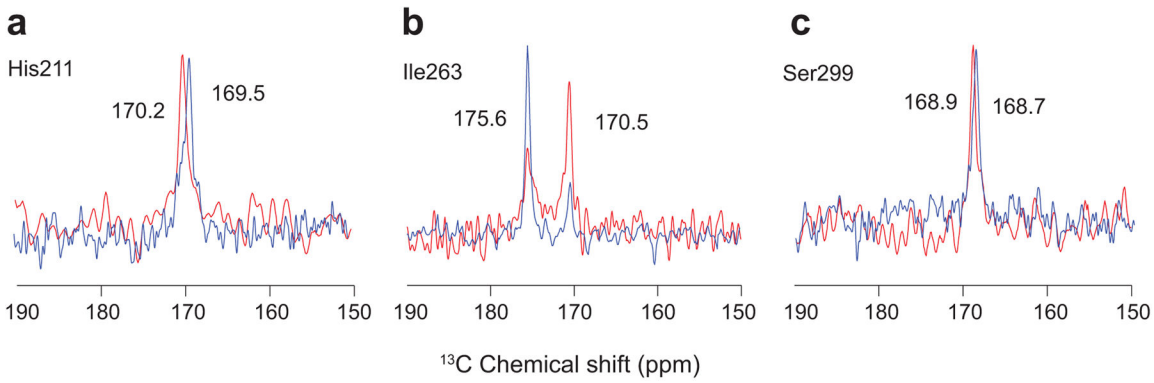

Figure 3.

$^{13}C…^{15}N$ REDOR NMR experiments of Meta II in the presence and absence of the Gα peptide of transducin. REDOR filtered spectra are shown for His211$^{5.46}$ (**a**), Ile263$^{6.46}$ (**b**) and Ser299$^{7.46}$ (**c**). The experiments on rhodopsin without (red) and with (blue) added Gα peptide were carried out in DDM micelles and mixed DDM/DOPS micelles, respectively.

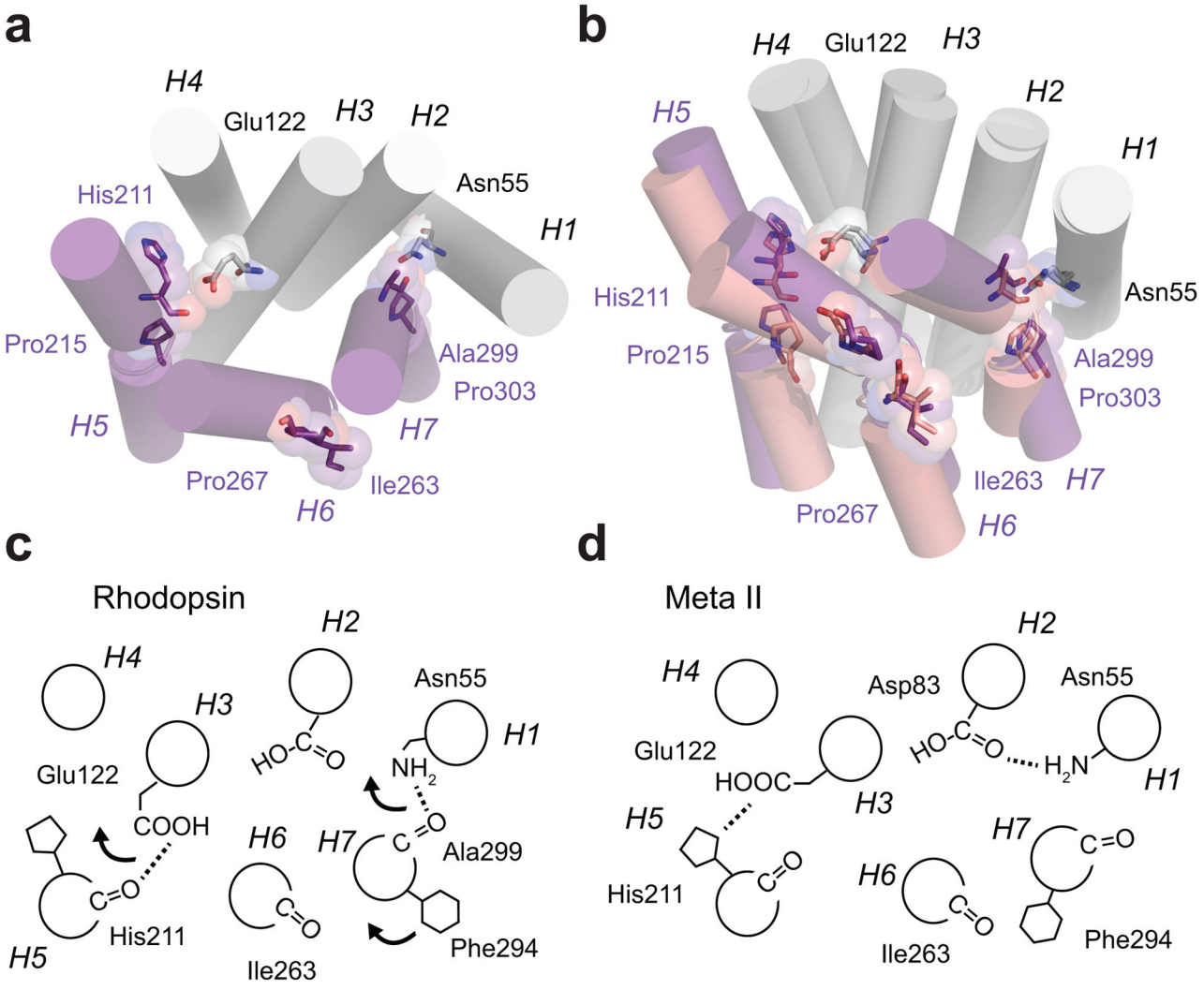

Figure 4.

Receptor activation leads to repacking of helices H5–H7 on the TM core of rhodopsin. (**a**) Inactive structure of rhodopsin. Helices H1–H4 form a scaffold onto which helices H5–H7 pack. The key packing contacts on H5 and H7 are associated with Pro215$^{5.50}$ and Pro303$^{7.50}$ and their corresponding *i-4* carbonyls. Retinal isomerization disrupts both interactions. For H5, the β-ionone ring has a steric clash at the position of the His211$^{5.46}$-Glu122$^{3.37}$ hydrogen bond upon conversion the all-*trans* configuration. For H7, the retinal is covalently attached to Lys296$^{7.43}$. Upon isomerization, Trp265$^{6.48}$ on H6 rotates away from H7 and disrupts a water mediated hydrogen bond with Asn302$^{7.49}$, which is part of a hydrogen bonding network stretching from Asn55$^{1.50}$ and Asp83$^{2.50}$ to the Ala299$^{7.46}$ C=O. (**b**) Overlap of the crystal structures of rhodopsin (1GZM[10], purple) and Meta II (3PQR[13], light purple) showing the positions of the TM helices. The disruption of the interactions of the His211$^{5.46}$ C=O and Ala299$^{7.46}$ C=O with the H1–H4 scaffold allows helices H5–H7 to

reorient. (**c,d**) Schematic of the hydrogen bonding changes occurring between inactive rhodopsin and active Meta II rhodopsin.

Table 1

NMR $^{13}C=O$ chemical shifts and crystal structure ϕ, ψ torsion angles in rhodopsin and Meta II

Residue	State	Chemical shift (ppm)[1]	ϕ[2]	ψ
His211	Rho Meta II	172.5 170.2[3]	***−115/−106***[4] ***−107***[4]	***53/22***[4] ***−3***[4]
Ile263	Rho Meta II	**171.9**[3] **170.5**/175.6	−55/−66 −48	−44/−41 −41
Ser299	Rho Meta II	173.1 **168.9**[3]	***−86/−82***[4] −65	***0/−8***[4] −20
Phe287	Rho Meta II	**169.3**[3] **169.3**[3]	−35/−59 −56	−60/−41 −34
Standard α-helix		175–177	−60	−45

[1] Rhodopsin and Meta II ^{13}C chemical shifts are taken from REDOR filtering experiments.

[2] Torsion angles are taken from the rhodopsin crystal structures (PDB ID 1U19[9] and PDB ID 1GZM[10]) and demarcated by slashes (1U19/1GZM). Torsion angles for Meta II are taken from the Meta II crystal structure (PDB ID 3PQR[13]).

[3] Low chemical shift values in bold are attributed to loss of or weaker C=O hydrogen bonding since the torsion angles are close to those for standard helices or, in the case of His211, the torsion angles become less distorted in Meta II.

[4] Torsion angles in bold and italics are distorted from those of standard α-helices.