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 stabilization of the receptor structure. To address the role of conserved prolines in family A GPCRs through solid-state NMR spectroscopy, we focus on bovine rhodopsin, a GPCR in the visual receptor subfamily. The free backbone C=O groups on helices H5 and H7 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, thus facilitating repacking of H5 and H7 onto the transmembrane core of the receptor. These results provide insights into the multiple structural and functional roles of prolines in membrane proteins.

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

To address the role of prolines in the structure and function of GPCRs, we focused on the visual receptor rhodopsin. Rhodopsin serves as an on-off switch for light detection in the vertebrate retina. Light energy absorbed by the retinal chromophore in rhodopsin serves as an on-off switch for light detection in the vertebrate retina. In the inactive state of rhodopsin (Metarhodopsin II), the free backbone carbonyls associated with Pro267 (C=O) and Pro291 (C=O) on helices H5 and H7 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, thus facilitating repacking of H5 and H7 onto the transmembrane core of the receptor. These results provide insights into the multiple structural and functional roles of prolines in membrane proteins.

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 contain a proline residue in the middle of the TM sequence: Pro215 (C=O), Pro267 (C=O) and Pro303 (C=O), respectively (residues are designated throughout the text according to the Ballesteros–Weinstein universal numbering system).

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 and Pro303 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 and Pro291 are oriented toward the membrane lipids and do not hydrogen-bond in the crystal structures of inactive rhodopsin or active opsin, thus instead suggesting 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, thus raising the possibility that the sequence stretching from Pro267 to Pro291, which includes extracellular loop EL3, pivots after activation.

Early FTIR spectroscopy studies on rhodopsin have indicated that conformational changes at one or more prolines occur after activation. FTIR difference spectra have revealed a large shift of the amide I vibration associated with a peptide bond adjacent to the N-terminal side of a proline. However, those studies have left open the possibility that the observed shift might be due to cis-trans proline isomerization. More recent studies using non-native amino acid substitutions at conserved prolines in the D2 dopamine receptor have ruled out that cis-trans proline isomerization occurs, at least in this specific GPCR. They have found that the main function of proline is 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 results in receptors with wild-type function. In agreement with this role, in several GPCRs mutation of proline to residues that are able to form backbone hydrogen bonds disrupts expression and/or function.

Solid-state NMR spectroscopy in combination with isotope labeling of rhodopsin can be used to clarify the roles 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, owing to the lack of an NH hydrogen-bond.

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Free backbone carbonyls mediate rhodopsin activation

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partner along their own helix backbone. Our focus was 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 with 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, which are part of a transmission switch that couples retinal isomerization to conformational changes in the intracellular side of the receptor$^{21,22}$. Finally, the Ala299$^{7.46}$ carbonyl associated with conserved Pro303$^{7.50}$ orients 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 three free carbonyls after receptor activation reveal that they play both structural roles in guiding the packing of the TM helices in the inactive receptor and 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 couples 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. Here, we used NMR spectroscopy 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 or 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=$^{15}$N resonance shifted to lower frequency after activation (Fig. 2a). The $^{13}$C=$^{15}$N resonance that changed chemical shift was assigned to His211$^{5.46}$ by using rotational echo double resonance (REDOR)$^{23}$ NMR filtering of rhodopsin specifically labeled with $^{[1-^{13}]$histidine and $^{[15]$N]phenylalanine. This labeling strategy generates a single $^{13}$C-$^{15}$N-labeled peptide bond because there is only a single histidine-phenylalanine pair in the rhodopsin sequence, namely His211$^{5.46}$-Phe212$^{5.47}$. The REDOR experiment allows measurement of $^{13}$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 selective observation of only the single $^{13}$C=O resonance in the REDOR filtered spectrum$^{23}$. Using REDOR filtering, we observed the $^{13}$C=O frequency of H211$^{5.46}$ at 172.5 p.p.m. in rhodopsin and at 170.2 p.p.m. after conversion to Meta II (Fig. 2a).

The decrease in the His211$^{5.46}$ $^{13}$C=O chemical shift in the transition to Meta II may have resulted 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 crystal structures. The chemical-shift changes associated with changes in backbone conformation versus hydrogen-bonding may be comparable in magnitude (4–5 p.p.m.)$^{24–26}$. 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 p.p.m. was lower than that normally observed for hydrogen-bonded C=O groups in α-helices, thus suggesting that the lower chemical shift is caused by nonhelical backbone torsion angles (bold, italics in Table 1). Indeed, in the crystal structure of rhodopsin, the $\phi$ and $\psi$ torsion angles show a strong distortion compared with those in standard α-helices ($\phi = -60^\circ$ and $\psi = -45^\circ$). After 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$^{11,27}$ (Fig. 2b). The direct His211$^{5.46}$-Glu122$^{3.37}$ interaction can already be sensed in the Meta I intermediate, as indicated through FTIR spectroscopy$^{28}$. 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), thus favoring 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 was in the opposite direction, and consequently we attributed it 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 $^{[1-^{13}]$isoleucine showed that several isoleucines exhibited different
chemical shifts after activation (Fig. 2c).

We took advantage of the unique Ile263\textsubscript{6.46} group observed in rhodopsin, alanine was scrambled and could not be specifically targeted. Therefore, to target this carbonyl, we mutated Ala299\textsubscript{7.46} to Ser299\textsubscript{7.46} using rhodopsin labeled with [1-13C]isoleucine and [15N]cysteine; and Ile263\textsubscript{6.46} (Fig. 2d) and toward the extracellular loop.

After activation, we found that the Ile263\textsubscript{6.46} resonance split into two components at 170.5 and 175.6 p.p.m. In the Meta II crystal structure\textsuperscript{12,13}, the backbone $\varphi$ and $\psi$ torsion angles were still close to values for standard $\alpha$-helices (Table 1). The upfield (170.5 p.p.m.) component of the Ile263\textsubscript{6.46} resonance was consistent with a non-hydrogen-bonding partner, because the backbone $\varphi$ and $\psi$ torsion angles of Ile263\textsubscript{6.46} are close to values observed in standard $\alpha$-helices.

Pro291\textsubscript{7.38}

The conserved Pro291\textsubscript{7.38} on helix H7 frees the carbonyl of Ala299\textsubscript{7.46} in rhodopsin. With the HEK293S expression system used for $^{13}$C-labeling rhodopsin, alanine was scrambled and could not be specifically labeled. Therefore, to target this carbonyl, we mutated Ala299\textsubscript{7.46} to Ser299\textsubscript{7.46}. The A299S rhodopsin mutant exhibited a 500-nm absorption band, and the photobleaching behavior and stability of Meta II were similar to those of the wild-type pigment (data not shown). The new Ser299\textsubscript{7.46} Val300\textsubscript{7.47} dipole sequence is unique. Using REDOR filtering on [1-13C]serine- and [15N]valine-labeled rhodopsin, we observed the Ser299 $^{13}$C=O resonance at 173.1 p.p.m. in rhodopsin and at 168.9 p.p.m. in Meta II (Fig. 2e).

The Ala299\textsubscript{7.46} C=O group is hydrogen-bonded to the side chain NH$_2$ of Asn55\textsubscript{1.50} (Fig. 2f). Like His211\textsubscript{5.46}, the backbone $\varphi$ and $\psi$ torsion angles of Ala299\textsubscript{7.46} in rhodopsin were different from those in standard $\alpha$-helices (Table 1), but in Meta II the values were consistent with those of $\alpha$-helices. We therefore attributed the large decrease in the chemical shift of the Ser299 $^{13}$C=O resonance after activation to a loss of hydrogen-bonding to Asn55\textsubscript{1.50} (Table 1). Given the high conservation of both Asn55\textsubscript{1.50} and the free C=O group at position 299 across the family A GPCRs, this structural change after activation is likely to be related to receptor function.

Pro291\textsubscript{7.38} at the extracellular end of H7 is highly conserved (82%) in the visual receptor subfamily, and it frees the carbonyl at Phe287\textsubscript{7.34}. There are two phenylalanine-methionine pairs, and correspondingly

| Residue | State | Chemical shift (p.p.m.) | $\varphi$ | $\psi$ |
|---------|-------|-------------------------|---------|-------|
| His211  | Rho   | 172.5                   | −115/−106$^4$ | 53/22$^4$ |
|         |       |                         | −107$^7$ | −3$^4$ |
| Ile263  | Rho   | 171.9$^3$               | −55/−66  | −44/−41 |
|         |       |                         | −48      | −41    |
| Ser299  | Rho   | 173.1                   | −86/−82$^4$ | 0/−8$^4$ |
|         |       |                         | −65      | −20    |
| Phe287  | Rho   | 169.3$^3$               | −35/−59  | −60/−41 |
|         |       |                         | −56      | −34    |
|         | Meta II | 169.3$^3$             | −56      | −34    |
| Standard $\alpha$-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, and values for PDB 1U19 (ref. 9) and PDB 1BZM, respectively, are shown separated by slashes. Torsion angles for Meta II are taken from the Meta II crystal structure (PDB 3PQR$^{13}$). $^3$Low chemical-shift values in bold are attributed to loss of or weaker C=O hydrogen-bonding because 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 $\alpha$-helices.
there were two peaks in the REDOR spectrum of rhodopsin at 174.1 p.p.m. and 169.3 p.p.m. (Fig. 2g). We assigned the 174.1 p.p.m. to Phe85 in helix H2. This chemical shift was consistent with the helical secondary structure and backbone hydrogen-bonding of Phe85 in the rhodopsin crystal structure. We assigned the 169.3 p.p.m. resonance to the Phe287 C=O on the basis of the Φ and Ψ torsion angles and the lack of a clear hydrogen-bonding interaction. The Phe287 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). After activation, we observed that the resonance at 174.1 p.p.m. split into two components at 174 and 175.3 p.p.m., and the resonance at 169 p.p.m. broadened. We attributed the broadening (and lower intensity) to increased disorder in the backbone structure at Phe287 C=O. We tentatively assigned the splitting of the 174.1 p.p.m. resonance to conformational changes occurring in this region of H6, we obtained REDOR NMR spectra of the free carbonyls at His211, Ile263, and Ser299 in the presence of the transducin Gα peptide.

Binding of the transducin Gα peptide to Meta II stabilizes the active structure (29). The C=O chemical shifts of His211, Ile263, and Ser299 did not move appreciably after Gα peptide binding (Fig. 3a–c). However, there was a marked increase in the intensity of the Ile263 C=O resonance with the unusual downfield chemical shift. The change in populations suggests that there is an induced fit of the Gα peptide into its intracellular binding site, which is allosterically coupled to the region containing the Ile263 C=O.

### DISCUSSION

Prolines are unique in lacking a backbone NH, thereby effectively eliminating the ability of the carbonyl group at the −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 −4 positions associated with these prolines are highly conserved as well. Because 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 allosteric activation.

### Induced fit of the C-terminal Gα peptide influences H6

The change in the 13C chemical shift of Ile263 after light activation was the most unusual among the three sites associated with the conserved prolines described above. A component of this 13C resonance shifted downfield in frequency; however, it has not been found to be 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 showed that the backbone torsion angles of Ile263 do not change appreciably after activation. To better understand the structural changes occurring in this region of H6, we obtained REDOR NMR spectra of the free carbonyls at His211, Ile263, and Ser299 in the presence of the transducin Gα peptide.

Figure 3 13C-15N REDOR NMR experiments of Meta II in the presence or absence of the C-terminal Gα peptide of transducin. REDOR filtered spectra are shown for His211 (15,46) (a), Ile263 (46) (b) and Ser299 (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.
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 break-
ing interhelical contacts and as such may mediate the conformational
changes that underlie protein function. Our study focused 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 exhibited several different hydrogen-bonding interactions and
consequently provided insights into the possible roles of prolines in
membrane proteins.

The structure of rhodopsin reflects the evolutionary requirements
of night vision: a photoreceptor that is finely tuned to balance low
dark noise (via a stable structure) and high sensitivity (through rapid
receptor dynamics after the absorption of a single photon). The first
four TM helices provide the stable framework onto which helices H5,
H6 and H7 pack in the dark-state structure. Helices H5, H6 and H7
each contain highly conserved prolines (Pro2155.50, Pro2676.50 and
Pro3037.50) and undergo the largest structural changes during activa-
tion (Fig. 4a, b). The free C=O groups associated with these prolines
are all approximately in the same plane within or near the receptor’s
TM core (Fig. 1). The TM core is highly conserved and is involved in
both receptor structure and function.

Structural roles are likely to exist for all three highly conserved
prolines. As the receptor folds during protein synthesis, the free car-
bonyls at His2115.46 and Ala2997.46 in the middle of the TM helices
are in a position to hydrogen-bond to the strongly polar Glu1223.37
and Asn551.50 side chains on helices H3 and H1, respectively.
Computational studies on receptor stability and unfolding have
highlighted His2115.46 and Ala2997.46 as the major TM residues that
contribute to the overall structural integrity of rhodopsin. 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
His2115.46 and Ala2997.46 were identified through two slightly dif-
f erent computational approaches, both results are consistent with
His2115.46 and Ala2997.46 being sites of autosomal dominant retinitis
pigmentosa (ADRP) mutations, which are typically associated with
protein misfolding. Mutation of Pro2676.50 also leads to protein mis-
fold ing and ADRP. Because the free C=O associated with Pro2676.50
does not hydrogen-bond in inactive rhodopsin, its structural role may
be to allow the helical segments on each side of Pro2676.50 to optimize
contacts with the other TM helices and possibly prevent non-native
(misfolded) interactions.

Functional roles of the three highly conserved prolines probably
involve conformational switching rather than conformational dynamics.
After activation, both the His2115.46 and Ser2997.46 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 they result in a rearrangement of interhelical
interactions and helix orientations when broken (Fig. 4c, d). The bal-
ance 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 an all-trans retinal SB (agonist) chromophore.
For H5, the new helix orientation is stabilized not by an alternative
hydrogen-bonding partner for the His2115.46 C=O but instead by the
formation of two new interhelical hydrogen bonds, one involving the
His2115.46 side chain with Glu1223.37, and the other involving the interaction of Tyr2235.58 with Arg1353.50. Conservative
mutations of either Glu1223.37 or Tyr2235.58 decrease the stability of
Meta II30, 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 Asn551.50 allows H7 to become more
helical. Mutations that favor a helical (nondistorted) conformation, such as the P303A mutant, lead to hyperactivity of the receptor.

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 has suggested that the free
C=O associated with conserved Pro4.60 in the β₁-adrenergic receptor
is part of a complex hydrogen-bonding network that links H4 and H5
and is modulated by ligand binding.

The observation of two distinct chemical shifts for Ile2634.6613C=O
resonance suggests that Pro2676.50 provides a hinge in H6 but that it is
not conformationally dynamic. The 13C=O resonances are sharp rather
than broadened, as observed for Phe2877.34. In agreement with this
idea, position 6.46 is 80% conserved as isoleucine, valine or leucine
and only 2% conserved as glycine. Glycine is thought to facilitate
dynamics when located at the i − 4 position relative to TM prolines.
The mixture of chemical shift changes of the Ile2634.6613C=O reso-

nance indicates that H6 does not toggle to a fully active conforma-
tion until binding of the Gα peptide. In contrast to the C=O groups
on H5 and H7, one component of the Ile2634.6613C=O resonance
shifts downfield. This observation is 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, thus suggesting that
H5 and H7 have adopted their fully active conformations before
G-protein binding.

Of the four prolines studied, only Pro2917.38 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 Pro2155.50 and Pro3037.50 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. Glycine occurs most often at position
5.46 (17%) in the family A GPCRs, and serine is the next highest
conserved at this position (11%). Olfactory receptors are excluded
from this analysis because 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 can
function as conformational switches in the conversion to the active
Meta II intermediate. As such, these findings are of broad impor-
tance in the structure and function of membrane proteins in general.
In a survey of high-resolution membrane-protein structures from the
Protein Data Bank, ~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

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appear in membrane proteins as diverse as transporters, enzymes and ion channels (Supplementary Note 2).

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** NMR data have been deposited in the Biological Magnetic Resonance Data Bank under accession code 26811.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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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. prepared samples; N.K., A.P. and S.O.S. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Expression and purification of 13C-labeled rhodopsin. Isotope-enriched bovine opsin was expressed with inducible HEK293S cell lines. The original cell lines were obtained from J. Nathans (Johns Hopkins University) but not authenticated or tested for mycoplasma contamination. HEK293S cells are widely used for production of recombinant proteins and viruses. Rhodopsin was generated from expressed opsin through incubation with ~30 µM 11-cis retinal, extracted from membranes with 1% (w/v) n-β-β-dodecyl maltopyranoside (DDM) in PBS, pH 7.4, and purified with Rho-1D4-Sepharose resin. 13C-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 nonamer elution peptide (TETSQVAPA) and concentrated 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 with a three-channel 4-mm MAS probe with a spinning rate of 10 kHz. Spectra were collected with a 2-ms contact pulse during cross-polarization. SPINAL64 decoupling was used during acquisition with a 1H RF field strength between 70 and 90 kHz. REDOR spectra were obtained with a dephasing period of 20 rotor cycles at a 10 KHz MAS rate (2 ms). The REDOR filtered spectra (ΔS) were obtained by subtracting spectra with (S) and without (S₀) rotor-synchronized 15N π pulses (10–11 µs). To reduce artifacts, S and S₀ spectral acquisition was interleaved, and difference spectra were acquired on a scan-by-scan basis. AS spectra were summed over 60,000–100,000 scans with ~5–6 mg rhodopsin in a typical sample.

For rhodopsin and Meta II spectra, states were cryotrapped at 190 K, as described previously. The spectra shown were generally repeated at least twice on samples purified from different cell growths without deviations of more than ±0.2 p.p.m. in the reported chemical shifts. The 13C high-resolution NMR and solid-state MAS NMR spectra were externally referenced to the 13C resonance of neat TMS at 0 p.p.m. at room temperature. With TMS as the external reference, we calibrated the carbonyl resonance of solid glycine at 176.46 p.p.m. The chemical-shift difference between 13C of DSS in D2O relative to neat TMS was 2.01 p.p.m.

Gα peptide synthesis and reconstitution with rhodopsin. The 15-mer C-terminal peptide of the Gα subunit of transducin (TDIIIKENLDCGLF) was synthesized with 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 out in mixed micelles with 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. DOPS was added to rhodopsin in DDM micelles in a 1:100 rhodopsin/lipid ratio, and the Gα peptide/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.

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