Highly conserved tyrosine stabilizes the active state of rhodopsin

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Light-induced isomerization of the 11-cis-retinal chromophore in the visual pigment rhodopsin triggers displacement of the second extracellular loop (EL2) and motion of transmembrane helices H5, H6, and H7 leading to the active intermediate metarhodopsin II (Meta II). We describe solid-state NMR measurements of rhodopsin and Meta II that target the molecular contacts in the region of the ionic lock involving these three helices. We show that a contact between Arg135, Met257, and Glu247 is formed in Meta II, consistent with the outward rotation of H6 and breaking of the dark-state Glu134–Arg135–Glu247 ionic lock. We also show that Tyr223 and Tyr306 form molecular contacts with Met257. Together these results reveal that the crystal structure of opsin in the region of the ionic lock reflects the active state of the receptor. We further demonstrate that Tyr223 and Ala132 to helix H5 in an active orientation. Mutation of Tyr223 to phenylalanine or mutation of Ala132 to leucine decreases the lifetime of the Meta II intermediate. Furthermore, the Y223F mutation is coupled to structural changes in EL2. In contrast, mutation of Tyr306 to phenylalanine shows only a moderate influence on the Meta II lifetime and is not coupled to EL2.

G protein-coupled receptor | solid-state NMR spectroscopy | ERY motif

Rhodopsin, the vertebrate photoreceptor for vision under dim light, belongs to the large, pharmaceutically important superfamily of G protein-coupled receptors (GPCRs). The photoreactive chromophore in rhodopsin is the 11-cis-isomer of retinal, which is covalently linked to Lys296 and Ser306. The structure of rhodopsin (2) reveals a tightly packed bundle of seven transmembrane (TM) helices but offers few clues as to how the helices move upon light activation.

Site-directed spin-labeling studies by Hubbell and coworkers (3, 4) showed that the largest change in the seven-TM-helix bundle involves an outward rotation of helix H6, consistent with an increase in volume of the receptor upon activation (5). The challenge for obtaining a high-resolution structure of the active metarhodopsin II (Meta II) intermediate has been that light activation causes the dark-state crystals of rhodopsin to dissolve (6), suggesting that the structural changes are sufficiently large to disrupt crystal packing. Salom et al. (7) were able to determine the crystal structure of 4.15 Å resolution of a photointermediate of rhodopsin containing retinal with a deprotonated Schiff base (SB) (7). The structure did not exhibit the large helix motions characteristic of the activated receptor, suggesting that this intermediate corresponds to the Meta II substate (Meta IIa) formed prior to helix motion (8).

More recently, Park et al. determined the structure of opsin (9). Opsin is formed when the Meta II intermediate decays and releases the agonist all-trans-retinal from the retinal-binding site. Opsin has low (≤1%) but detectable, basal activity in rod outer segment cell membranes (10). At pH 4, FTIR difference spectra of opsin exhibit vibrational bands characteristic of Meta II (11), suggesting that opsin adopts an active conformation. The crystals of opsin obtained at pH 6 appear to retain many features characteristic of the active state (Fig. 1). In fact, the most recent crystal structure of opsin (12) contains the bound C-terminal peptide of the θ subunit of transducin in a conformation similar to that observed in solution NMR studies on the activated Meta II intermediate (13, 14).

One of the most striking features of the opsin structure is that the ionic lock involving Glu134–Arg135–Glu247 of the conserved ERY sequence on H3 and Glu247 on H6 is disrupted (Fig. 1B). The opsin structure reveals an outward motion of H6, similar in magnitude (~6 Å) to the change observed by spin-labeling studies (3). Arg135 is extended towards Met257, Tyr223, and Tyr306. Both Tyr223 and Tyr306 have strong sequence identity in the class A GPCRs. Tyr306 is part of a cluster of highly conserved residues on the cytoplasmic side of H7 and is thought to impart stability of the inactive receptor (15). Tyr223 is unusual in that it is situated away from the helical bundle in the inactive, dark receptor but rotates in toward the ionic lock in the opsin crystal structure. Whereas Tyr306 of the NPxxY sequence has been characterized extensively, the only evidence that Tyr223 plays a critical role in receptor activation comes from the opsin structure.

Solid-state NMR spectroscopy provides a unique way of following the structural transitions from rhodopsin to Meta II (16, 17) by allowing measurements in a fluid membrane environment, which facilitates the conformational changes associated with the formation of Meta II. The work to date has focused on the retinal-binding pocket. Here, we use solid-state NMR to determine the structure of the ionic lock in Meta II and fluorescence spectroscopy to characterize the rates of Meta II decay in mutants of rhodopsin where three highly conserved tyrosines (Ty316, Ty223, and Ty306) are replaced with alanine.


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provide insights into the roles of these three conserved tyrosines. We show that Tyr223 ε58 has a more substantial contribution to the stability of Meta II and transducin activation than either Tyr136 ε58 or Tyr306 ε58.

Results

The Open State of the Ionic Lock in Meta II. The outward rotation of H6 observed in the opsin crystal structure places the side chain of Met257 δ40 in close proximity to Arg135 ε50. The 13N chemical shifts of the Nζ1, Nη2, and Nε nitrogen atoms of the 13C chemical shift of the Cζ carbon were measured to address the changes in the environment of Arg135 ε50 upon Meta II formation. The arginine 15N chemical shifts are sensitive to the protonation state and environment of the guanidinium group (18), and one can envisage that when the interactions between Glu134 δ49, Arg135 ε50, and Glu247 ε50 are broken, Arg135 ε50 undergoes a change in electrostatic environment.

The 13N and 13C spectra of arginine-labeled rhodopsin and Meta II are presented in Fig. S1. There is no chemical shift resolution in the 15N resonances of the arginine Nζ1, Nη2, and Nε nitrogens in either rhodopsin or Meta II, and there is only a slight (<1 ppm) shift in the 13Cζ resonance. The lack of significant changes indicates that the protonation state of Arg135 ε50 has not changed and suggests that the electrostatic environment surrounding Arg135 ε50 is similar in the inactive and active states. These results suggest that either there are no large changes in the receptor structure as suggested by the crystal structure of the rhodopsin photoprotein with a deprotonated retinal SB or that disruption of the full charge–charge interactions within the Glu134 δ49, Arg135 ε50, Glu247 ε50 ionic lock are compensated by partial charge interactions that result in no substantial change in the 15N or 13C chemical shifts of Arg135 ε50.

In contrast to the lack of chemical shift changes in arginines, there are substantial changes observed in the chemical shifts of methionines. Fig. 2A presents the 13C NMR difference spectrum between 13Cζ-Met-labeled rhodopsin and Meta II. The negative 13C δ50 resonance at 155 ppm can tentatively be assigned to Met257 δ40 in Meta II on the basis of its sensitivity to mutation of Tyr223 δ58 and Tyr306 δ53. To test for a direct Arg135 δ50, Met257 δ40 interaction in Meta II, we labeled rhodopsin with U-13C δ50, Arg135 δ50, and measured internuclear Arg-Met distances by using 2D 13C dipolar-assisted rotational resonance (DARR) NMR. We do not observe Arg135 δ50-Met13C δ50 crosspeaks in the 2D DARR NMR spectrum of rhodopsin (Fig. 2B, black line) consistent with the rhodopsin crystal structure where no Arg135 δ50-Met13C δ50 carbon pairs are closer than 6 Å. In contrast, an Arg135 δ50-Met13C δ50 crosspeak is observed in Meta II (Fig. 2B, red line) that we tentatively assign to Arg135 δ50, Met257 δ40. In the opsin crystal structure, the Arg135 δ50, Met257 δ40 distance is 4.6 Å, within the range of this experiment. The putative Arg135 δ50, Met257 δ40 contact in Meta II correlates with the position of these two residues in the opsin crystal structure. The next closest Arg-Met pair is Arg135 δ50 and Met256 δ56, whose Cζ-Cε distance is 6.5 Å.

To further characterize the local environment of Arg135 δ50 in Meta II, we targeted the two conserved tyrosines (Tyr223 δ58 and Tyr306 δ53) that appear to stabilize the ionic lock in an open conformation (Fig. 1B). The side-chain hydroxyl groups of Tyr223 δ58 and Tyr306 δ53 may act in concert to preserve the dense-state electrostatic environment of Arg135 δ50.

Fig. 3 presents difference spectra generated by subtraction of the spectrum of wild-type rhodopsin from the spectrum of one of three tyrosine mutants (Y136F, Y223F, or Y306F). Only the region of the 13C δ50 tyrosine resonances is shown. These spectra allow us to directly assign the 13C δ50 tyrosine chemical shifts in rhodopsin and in Meta II. Both the Tyr223 δ58 and Tyr306 δ53 resonances shift downfield slightly upon activation to 156.2 and 155.9 ppm, respectively, reflecting an increase in hydrogen bonding of the 13C δ50, OH group and indicating that both tyrosines are protonated and in a similar environment in Meta II. For comparison, the difference spectrum of Y136F is shown in Fig. 3A. The 13C δ50 chemical shift of Tyr136 δ51 does not change upon activation.
In rhodopsin, rows taken through the Met-C mutants. With a native-like inactive conformation being adopted by both Met (17). In the dark, both Tyr change upon substitution of Tyr the second extracellular loop (EL2) deep within the retinal-bind-network of hydrogen-bonding interactions that help to position an interaction between Trp rhodopsin, fluorescence increases in the transition from Meta II in wild-type rhodopsin and the Y223F mutant. For wild-type decay of Meta II by fluorescence spectroscopy. To address that these residues interact with one another and contribute to (Fig. S1) suggests that the electrostatic environment around both Tyr residues interact with one another and contribute to the stabilization of the active Meta II conformation. To address that these residues interact with one another and contribute to the stabilization of the active Meta II conformation. To address that these residues interact with one another and contribute to the stabilization of the active Meta II conformation. To address that these residues interact with one another and contribute to the stabilization of the active Meta II conformation. To address that these residues interact with one another and contribute to the stabilization of the active Meta II conformation. To address that these residues interact with one another and contribute to the stabilization of the active Meta II conformation.

Fig. 4. Tyr223S,58, Met257S,40 and Tyr306S,53, Met257S,40 contacts occur in Meta II. Rows through the Met-C diagonal resonance are shown from 2D 13C DARR NMR spectra of rhodopsin (black line) and Meta II (red line) labeled with 
13C-tyrosine and 13C-methionine. The rhodopsin and Meta II rows are overlaid with the corresponding rows obtained of the Y223F (blue line) and Y306F (green line) mutants in order to determine if either of these tyrosines contact Met257S,40 in Meta II. Upon mutation of Tyr223S,58 or Tyr306S,53, there is loss of intensity of the crosspeak at 156 ppm, consistent with these tyrosines having similar chemical shifts and interacting with Met257S,40 in Meta II.

To establish if direct Tyr223S,58, Met257S,40 and Tyr306S,53, Met257S,40 contacts occur in Meta II, we obtained 2D DARR NMR spectra of wild-type (black line) and mutant (red line) rhodopsin 13C labeled at 13C-tyrosine and 13C-methionine (Fig. 4). In rhodopsin, rows taken through the Met-C diagonal resonance of the DARR spectrum reveal crosspeaks between Tyr191EL2, Met288EL2 at 156.8 ppm and Tyr268EL2, Met288EL2 at 155.2 ppm (17). In the dark, both Tyr191EL2 and Tyr268EL2 participate in a network of hydrogen-bonding interactions that help to position the second extracellular loop (EL2) deep within the retinal-binding pocket. The observation that neither of these crosspeaks change upon substitution of Tyr223S,58 or Tyr306S,53 is consistent with a native-like inactive conformation being adopted by both mutants.

In the rows corresponding to wild-type Meta II, a shoulder appears at ∼156 ppm (Fig. 4 B and D, red line), consistent with the chemical shifts of Tyr223S,58 and Tyr306S,53 observed in Fig. 3. This shoulder is lost upon mutation of either Tyr306S,53 or Tyr223S,58. These data confirm the proximity of Met257S,40 to both Tyr306S,53 and Tyr223S,58 in Meta II. In turn, these results place both tyrosine side chains in close proximity to the active state position of Arg135S,50.

Tyr223S,58 Stabilizes the Active Meta II Intermediate. The lack of chemical shift changes in the arginine 15N and 13C resonances (Fig. S1) suggests that the electrostatic environment around Arg135S,50 does not change appreciably upon activation. This observation along with the NMR structural data on Meta II showing that Tyr223S,58 and Tyr306S,53 surround Arg135S,50 suggests that these residues interact with one another and contribute to the stabilization of the active Meta II conformation. To address the role of these tyrosines in Meta II stability, we monitored the decay of Meta II by fluorescence spectroscopy.

Fig. 5 presents fluorescence data on the decay of Meta II in wild-type rhodopsin and the Y223F mutant. For wild-type rhodopsin, fluorescence increases in the transition from Meta II to opsin. The fluorescence changes are associated with a loss of an interaction between Trp265S,58 and the retinal chromophore that quenches tryptophan fluorescence in Meta II (19). When plotted as a function of time after illumination, the fluorescence increase can be fitted to a single exponential function. For the Y223F mutant, the fluorescence intensity reaches a maximum after 400 s corresponding to a fivefold increase in the Meta II decay relative to the wild-type receptor. For comparison, mutation of either Tyr136S,51 or Tyr306S,53 to phenylalanine increases Meta II decay by less than a factor of 2 (Table 1). These data suggest that Tyr223S,58 has a greater contribution to Meta II stability than either Tyr136S,51 or Tyr306S,53.

We tested the ability of the three tyrosine mutants (Y136F, Y223F, and Y306F) to activate the G protein transducin (Table 1). For the Y223F mutant, there is a large decrease in both the initial rate of transducin activation and the maximum level of activation as compared to wild-type rhodopsin (as revealed by the normalized fluorescence increase, Table 1), consistent with the rapid hydrolysis of the all-trans-retinal SB in the formation of opsin.

For the Y136F mutant, we observed that Meta II stability was similar to wild-type rhodopsin. However, the initial rate of transducin activation was significantly (1.5×) higher. In a previous study, it was found that a nearby rhodopsin retinitis pigmentosa mutant (Y137M) also displayed elevated (1.25×) initial rates of transducin activation (20). In the case of Y136F, we do not observe an associated increase in SB hydrolysis, suggesting that the structural consequences of this mutation are limited to the cytoplasmic side of the receptor. The analogous tyrosine has been the focus of several studies in other class A GPCRs, such as the vasopressin receptor (21) and CCR3 (22), where a conservative substitution blocks signaling while maintaining native-like ligand affinity.

Further support for the role of Tyr223S,58 in stabilizing Meta II comes from FTIR measurements obtained at lower temperature (Supporting Information). Analysis of vibrational band intensities characteristic of Meta I and Meta II shows that the Meta I ↔ Meta II equilibrium is strongly shifted to the Meta I state in the Y223F mutant (Fig. S2).

Table 1. The normalized rates of Meta II decay and transducin activation of Tyr136S,51, Tyr223S,58, Tyr306S,53, and Ala132S,57 mutants at 20 °C

<table>
<thead>
<tr>
<th>Meta II decay (n = 3)</th>
<th>Transducin activation (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Half-life, min</td>
</tr>
<tr>
<td>Wild-type</td>
<td>14.8 ± 0.3*</td>
</tr>
<tr>
<td>Y136F</td>
<td>12.5 ± 0.9</td>
</tr>
<tr>
<td>Y223F</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>Y306F</td>
<td>10.5 ± 0.6</td>
</tr>
<tr>
<td>A132S</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>A132S</td>
<td>9.3 ± 1.2</td>
</tr>
</tbody>
</table>

*Mean ± standard error.
Glu

modulate SB hydrolysis (27), including Glu on or near EL2 on the extracellular side of rhodopsin that can be defined by hydrolysis of the SB. There are several amino acids that are allosterically coupled to the extracellular side of the receptor. This hypothesis is in agreement with the coupling of H5 motion during activation (17). Meta II decay is associated with Cys and SB hydrolysis.

Y223F mutant suggest that the changes introduced by this mutation are pairwise stabilizing interaction between Tyr223 and Ala132 on or near EL2 on the extracellular side of rhodopsin that can modulate SB hydrolysis (27), including Glu113 and SB hydrolysis.

To characterize whether the mutation of Tyr223 or Tyr306 is coupled to structural changes in EL2, we expressed the Y223F and Y306F mutants with Cys-labeled cysteine. The cysteine Cβ-spectrum provides a probe for changes in the highly conserved Cys10 and Cys187 disulfide bond. The β-carbon resonances in cysteines are observed in a unique chemical shift window (34–50 ppm) and are sensitive to the secondary structure: 34–43 ppm for α-helices and 36–50 ppm for β-sheets. We previously observed strong crosspeaks between the Cys10 and Cys187 disulfide bond resonances at 36.4 and 46.8 ppm in the dark, respectively (17). These resonances do not change position in the Y223F or Y306F rhodopsin spectra.

Fig. 6 presents rows from the 2D DARR NMR spectra of Meta II of the Y223F (blue line) and Y306F (green line) mutants labeled with Cys-labeled cysteine. In contrast to the comparison with dark rhodopsin, there is a shift of the Cys187 resonance in the Meta II spectrum of the Y223F mutant compared to wild-type Meta II. In wild-type Meta II, the Cys187 resonance shifts to 50.1 ppm because of a change in the conformation of EL2, whereas the chemical shift of Cys110 on helix H3 does not change (17). In the Y223F mutant, the Cys187 resonance moves upfield to 48.2 ppm (Fig. 6A). Fig. 6C shows the crosspeak associated with Cys110 and Cys187. This crosspeak does not shift in the Y223F mutant, confirming that the influence of the mutation is localized to EL2. In the Y306F mutant (Figs. 6B and D), the crosspeaks associated with Cys110 and Cys187 are at the same position as in wild-type Meta II, indicating that there is no coupling between the NPxxY sequence and the conserved cysteine disulfide.

Discussion

The current study addresses several open questions regarding how retinal isomerization on the extracellular side of rhodopsin is coupled to the cytoplasmic ionic lock. We show that the interactions of Arg135, Met257, Tyr223, and Tyr306 observed in the opsin crystal structure are also present in the active Meta II intermediate, consistent with the outward rotation of H6 (9, 12). Mutational studies indicate that the Arg135, Tyr223, and Tyr306 interaction, which is facilitated by group-conserved Ala1324,7, has a strong influence on the stability of the active state conformation.

Defining the Open and Closed States of the Glu134/Arg135, Glu247/30 Ionic Lock. The ionic lock was originally described as a conserved hydrophobic cage motif in the gonadotropin-releasing hormone receptor at the cytoplasmic end of H3 involving Asp39, Arg30, and Ile34 (29). Ballesteros et al. (29) proposed that a salt bridge between Asp39 and Arg30 stabilizes the inactive receptor and that upon activation, Asp39 becomes protonated with the charged Arg30 side chain being prevented from orienting toward the cytoplasmic surface by Ile34. A more complex ionic lock involving the interaction of Arg30 with both Asp39 and Glu30 was based on the observation of increased basal activity in the D3.49N and E6.30Q mutants of the β3 adrenergic receptor (30). In the past few years, however, the crystal structures of the adenosine A2a (31) and β1 (32) and β2 (33) adrenergic receptors have been determined and, in contrast to rhodopsin, show no direct interaction between Arg30 and Glu30, although the Asp39–Arg30 salt bridge is retained.

Our results provide insights into the nature of the closed and open states of the ionic lock. Table S1 presents a summary of the conservation of the residues contributing to the ionic lock in rhodopsin and Meta II. There is a strong conservation of position 3.47 has the highest level of group conservation within this group (99%). Position 3.47 has a high propensity to mediate TM-helix interactions (28). We propose that the position of EL2 is linked to the position of Glu113 and SB hydrolysis.
indicate that, rather than stabilizing the active state, Met
residues observed at position 6.40. When substituted into rhodop-
β
Glu
described above, in combination with the opsin structure, provide
receptors, the position of Arg
subfamily this site is predominantly a serine (68% identity). In
in the rhodopsin subfamily of class A GPCRs. In the amine
in the rhodopsin subfamily of class A GPCRs. In the amine
in the rhodopsin subfamily of class A GPCRs. In the amine
respectively, on EL2. Coupling of the position of
58
and Arg
in the active Meta II intermediate can explain both the high
the β2-adrenergic receptor, we previously studied the influence
absorption of wild-type Ala at position 3.47 with Leu or Val dramatically
lowed receptor activity, whereas Ser at position 3.47 exhibited
wild-type activity, suggesting that the role of Ala132
in rhodop-
likely is the same across the class A GPCRs. In the recent
structure of both the β2-adrenergic and A2a receptors, Tyr
has rotated toward Ala
, as in activated opsin. This
active orientation for Tyr
may be due to the T4 lysozyme (T4L) insert between H5 and H6 used to crystallize both recep-
comparison with the wild-type receptors shows that the T4L
insert results in higher affinity for subtype-selective agonists,
which the authors suggest may reflect a shift toward the active
state (31, 33). In addition, MD simulations of the β2 receptor
without the T4L insert show rapid formation of the Arg
-Glu
interaction (34). In the β1 receptor, which was not crystallized
with the T4L insert, Tyr
was mutated to alanine as part of a
suite of mutations engineered to stabilize the inactive conforma-
tion of the receptor (32).
Table S2 summarizes the conservation of residues stabilizing
the active conformation of Arg135
. The conservation ofTyrc23
,83
is striking because it is oriented toward the lipid in
the dark state of rhodopsin, implying that it has only protein
contacts in the active state. In contrast, Met at position 6.40 is
not conserved in the class A GPCRs yet plays an important
role in stabilizing the inactive conformation of rhodopsin (see
below). Together, these observations support the view that
the Glu134
-Arg135
salt bridge is the essential interaction for
stabilizing the inactive state of class A GPCRs (36). Neutralization
of Glu134
, rather than breaking of the Arg135
salt bridge, is key to shifting the receptor to its active
conformation where the conserved interactions are between Arg3
, Tyr5
, and Tyr7
, as observed in Meta II and the crystal
structure of opsin.
Met257
Stabilizes Rhodopsin in the Inactive State. We had previ-
ously observed that most site-directed mutants of Met257
allow opsin activation by the addition of all-trans-retinal as a
diffusible ligand (37). The observation that Met257
shifts into contact with Arg135
 in the H3–H6 interface provides an
explanation for the Met257
mutations and the role of Met257
in activation. The Met257
mutants with the highest constitutive activity are M257Y, M257N, and M257S. The polar side chains at position 6.40 in these mutants interact more strongly with Arg135
 than the hydrophobic Met side chain and consequently
stabilize the active state. In other class A GPCRs (Table S2), the
β-branched amino acids (Thr, Ile, and Val) are the most common
residues observed at position 6.40. When substituted into rhodop-
(37), these residues do not confer appreciable constitutive
receptor activity (4.4–9.6%) but do allow almost full activation
(62–83%) upon the addition of all-trans-retinal. These results
indicate that, rather than stabilizing the active state, Met257
stabilizes the inactive state of the receptor. If Met257
is not
stabilizing the Meta II structure, the question arises as to the
relative stabilizing effects of Tyr223
 and Tyr306
. When
these two tyrosines are mutated individually to phenylalanine,
our measurements of transducin activation and Meta II decay
indicate that Tyr223
plays a much greater role in stabilizing
Meta II than Tyr306
.
Ala132
Serves as a Molecular Notch for Tyr223
. In the class A
GPCRs, there are 13 group-conserved residues that are located
mainly in the interfaces between helices H1–H4 (24). Import-
tantly, Ala132
 does not fit this pattern. Ala132
 is located on
H3 but oriented toward the H5–H6 interface. The results
described above, in combination with the opsin structure, provide
an explanation for the high group conservation (99%) of
this residue. Whereas the group-conserved amino acids mainly
mediate helix–helix interactions in the inactive state of rhodopsin,
Ala132
acts as a molecular notch to orient the Tyr223
-side
chain efficiently toward Arg135
and stabilize the active Meta
II intermediate (Fig. 1).
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Ala132
,67 serves to stabilize the active orientation for Tyr223
and Tyr306
. When
these two tyrosines are mutated individually to phenylalanine,
our measurements of transducin activation and Meta II decay
indicate that Tyr223
plays a much greater role in stabilizing
Meta II than Tyr306
.
Methods
Materials. 13C-labeled amino acids were purchased from Cambridge Isotope
Laboratories.
Expression and Purification of 13C-Labeled Rhodopsin. Stable tetracycline-indu-
cible HEK293S cell lines containing the opsin (bovine) gene and its mutants
was used to express rhodopsin. The expression and purification in n-dodecyl
maltoside (DDM) have previously been described (16, 17).
Transducin Activity. The reaction mixture containing mutant rhodopsin
(20 nM) and transducin (250 nM) in 10 mM Tris, pH 7.2, 2 mM MgCl
, 100 mM NaCl, 1 mM dithiothreitol, and 0.012% (wt/vol) DDM was illumina-
ted by using a 495-nm long pass filter at 20 °C with constant stirring.
The reaction was initiated by the addition of 5 μM GTPγS, and the fluores-
cence was monitored for 2,000 s. The sample was excited at 295 nm (2 nm

bandwidth) and emission was monitored at 340 nm (15 nm bandwidth) at 3-s intervals with an integration time of 2 s. Initial rates were calculated by using the data points collected over the first 60 s following GTP addition.

Fluorescence Spectroscopy. Meta II decay was monitored by using a fluorescence-based assay (19). Rhodopsin (250 nM) was illuminated by using a 495-nm long pass filter at 20 °C in 10 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane, pH 6 containing 0.1% (wt/vol) DDM. The fluorescence was monitored every 30 s for 7,200 s by using a 2-s integration time. Samples were excited at 280 nm (2 nm bandwidth) and emission measured at 330 nm (15 nm bandwidth).

Solid-State NMR Spectroscopy. Solid-state \(^{13}\)C magic angle spinning spectra were acquired at a static magnetic field strength of 14.1 T (600 MHz) on a Bruker AVANCE spectrometer as previously described (16, 17). NMR spectra were obtained at 190 K by using rhodopsin or Meta II solubilized in DDM.

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Supporting Information

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Fig. S1. ¹⁵N (A) and ¹³C (B) chemical shifts as a probe of the environment and protonation state of Arg135. It is thought to be the only arginine that experiences an appreciable change in environment upon photoactivation. Magic angle spinning NMR spectra illustrate that the ¹⁵N and ¹³C chemical shifts of the guanidinium group of arginine do not change appreciably between rhodopsin (black line) and metarhodopsin II (Meta II) (red line). The arginine Nη₂ resonances of Meta II appear to overlap those seen in rhodopsin, indicating that Arg135 remains protonated and is in a similar hydrogen-bonding environment despite data supporting neutralization of the ionic lock upon receptor activation (1, 2). Petkova and coworkers (3) found that the guanidinium nitrogen atoms of the protonated arginine side chain are generally involved in hydrogen-bonding interactions and exhibit chemical shifts ranging from 68.9 to 56.3 ppm for the δ-nitrogen, from 62.0 to 45.6 ppm for the downfield η resonance, and from 46.8 to 34.2 ppm for the upfield η resonance. The differences in the Nη chemical shifts are attributed to asymmetry in hydrogen bonding and can be almost as large as the >40-ppm differences observed upon deprotonation (4). The NMR data presented here along with the crystal structure of opsin (5) indicate that the protonated Arg135 side chain is stabilized in a symmetric fashion by hydrogen-bonding interactions with Glu134, Tyr225, and Tyr306. The chemical shift measurements of arginine and tyrosine indicate that Arg135 is charged and that Tyr225 and Tyr306 are neutral. In the crystal structure of opsin, the Gα peptide is observed to bind in a helical conformation with the negatively charged C terminus most closely associated with Lys311 on H8 of rhodopsin. Gα binding does not appear to be driven by the electrostatic environment created by a protonated arginine side chain.

Fig. S2. FTIR studies of wild-type rhodopsin and the Y223F and A132L mutants. FTIR spectroscopy provides a complementary method to NMR and absorption spectroscopy for characterizing the activation of rhodopsin. The FTIR-based titration curves (Θ_{FTIR}) of the Meta I to Meta II equilibrium are shown on the basis of monitoring the vibrational bands characteristic of Meta I and Meta II as a function of pH (1). It has previously been shown that coupling of the two protonation switches that control receptor activation at 10 °C and below reestablishes the classical two-state equilibrium between Meta I and protonated Meta IIbH+ (1). Here, we use FTIR to probe how the Y223F and A132L mutations influence this equilibrium. Mutation of Tyr223 to phenylalanine causes a strong shift in the equilibrium toward the Meta I conformation. The observation of a shift of the equilibrium toward Meta I in the Y223F mutant highlights the role of this tyrosine in stabilizing the active state of rhodopsin. In contrast, mutation of Ala132 to leucine does not shift the overall Meta I to Meta II equilibrium. However, the A132L mutant exhibits an increased alkaline endpoint, consistent with a shift from Meta I to Meta IIb (1). The FTIR spectrum of the Meta II intermediate of the A132L mutant shows changes in the range between 1,640 and 1,700 cm\(^{-1}\) that resemble those seen in Y223F. Within this range are the amide I vibrations and the strongest vibrations of the arginine guanidium side chain. Together with the Meta II decay and transducin activation data in Table 1, these results indicate that mutation of Ala132 influences both the Meta I and Meta II intermediates. FTIR spectroscopy was performed with a Bruker Vertex 70 spectrometer with a mercury-cadmium-telluride detector. Spectra were recorded at 4 cm\(^{-1}\) using 200–400 pmol membrane-reconstituted pigment and the sandwich sample type with 200 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (at pH 6.0 and higher) or MES buffer (at pH 5.0) (1). Photoactivation was achieved by a 1-s photolysis using an array of six light-emitting diodes at 530 nm (1). Spectra were normalized by using the fingerprint bands of the chromophore between 1,200 and 1,300 cm\(^{-1}\). Membrane reconstitution of n-dodecyl maltoside (DDM)-purified pigments was achieved by mixing pigment and phosphatidylcholine lipids from egg yolk (from a 6.3 mM stock in 1% DDM) at a protein:lipid molar ratio of 1:200. The mixture was incubated for 2 h on ice. Detergent was extracted by incubation with small polystyrene beads (Biobeads SM-2; BioRad), which were washed extensively with water to remove glycerol and floating beads. DDM extraction was performed in two rounds of incubation of 4 h and a last round of 12 h at 4 °C on a rotator. For each milligram of DDM present (prior to the first round of extraction), 20 mg of washed biobeads were used in each round, ensuring complete removal of the detergent. After each round, biobeads were allowed to settle to recover the supernatant. After the last round of extraction, all biobeads were carefully removed and proteoliposomes were collected by centrifugation for 4 h at 100,000 \(\times\) g.


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Table S1. Conservation of Ala\textsuperscript{3.47}, Glu\textsuperscript{3.49}, Arg\textsuperscript{3.50}, Tyr\textsuperscript{3.51}, Val\textsuperscript{3.54}, Tyr\textsuperscript{5.58}, Glu\textsuperscript{6.30}, Met\textsuperscript{6.40}, and Tyr\textsuperscript{7.53}

<table>
<thead>
<tr>
<th>Class A Rhodopsin ( \beta_{2})-AR</th>
<th>Percent occurrence*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Identity</td>
</tr>
<tr>
<td>3.47 Ala</td>
<td></td>
</tr>
<tr>
<td>3.49 Asp</td>
<td></td>
</tr>
<tr>
<td>3.50 Arg</td>
<td></td>
</tr>
<tr>
<td>3.51 Tyr</td>
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</tr>
<tr>
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</tr>
<tr>
<td>5.58 Tyr</td>
<td></td>
</tr>
<tr>
<td>6.30 Glu</td>
<td></td>
</tr>
<tr>
<td>6.40 Ile</td>
<td></td>
</tr>
<tr>
<td>7.53 Tyr</td>
<td></td>
</tr>
</tbody>
</table>

*Analysis is based on the class A G protein-coupled receptors (GPCRs) (~10,300 sequences) excluding the large olfactory receptor subfamily, which contains roughly 50% of the class A GPCR sequences. The sequence identities listed in parentheses include the olfactory receptors. Inclusion of the olfactory receptors has the potential to bias the results for several of the positions discussed (e.g., position 3.47 is predominantly alanine in the olfactory receptors). Note that phenylalanine is included in aromatics and hydrophobics, and histidine is considered polar only.

Table S2. Absolute occurrence in class A GPCRs

| Amino acid | Ala | Arg | Asn | Asp | Cys | Gln | Glu | Gly | His | Ile | Leu | Lys | Met | Phe | Pro | Ser | Thr | Trp | Tyr | Val |
|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 3.47       | 3656 | 5   | 30  | 4   | 90  | 1   | 0   | 157 | 26  | 9   | 13  | 3   | 1   | 4   | 5098 | 1194 | 1   | 2   | 5   |
| 3.49       | 39   | 20  | 245 | 6   | 697 | 4   | 177 | 2607 | 32  | 126 | 18  | 14  | 8   | 7   | 46  | 2   | 14  | 101 | 61  | 17  | 67  |
| 3.50       | 3    | 10,017 | 4 | 0   | 69  | 17  | 4   | 5   | 23  | 6   | 33  | 90  | 5   | 5   | 2   | 4   | 3   | 7   | 6   |
| 3.51       | 43   | 16  | 34  | 0   | 670 | 52  | 4   | 18  | 398 | 6   | 48  | 5   | 16  | 920 | 4   | 97  | 7   | 789 | 141 | 36  |
| 3.54       | 128  | 3   | 28  | 4   | 4   | 0   | 4   | 16  | 0   | 5,585 | 441 | 4   | 171 | 33  | 1   | 10  | 142 | 0   | 1   | 2   | 3,722 |
| 5.58       | 31   | 7   | 444 | 4   | 73  | 55  | 1   | 21  | 124 | 51  | 52  | 7   | 3   | 61  | 5   | 357 | 99  | 5   | 8,869 | 43  |
| 6.30       | 144  | 1,537 | 281 | 636 | 58  | 219 | 3,425 | 195 | 200 | 30  | 301 | 1,819 | 129 | 38  | 131 | 617 | 351 | 18  | 65  | 97  |
| 6.40       | 187  | 5   | 55  | 0   | 56  | 6   | 5   | 17  | 36  | 2,988 | 1,427 | 17  | 1,117 | 138 | 8   | 70  | 575 | 2   | 49  | 3,528 |
| 7.53       | 10   | 0   | 12  | 17  | 94  | 1   | 6   | 10  | 17  | 9   | 155 | 10  | 1   | 252 | 5   | 15  | 21  | 5   | 9,255 | 7   |

*Analysis is based on the class A GPCRs (~10,300 sequences) excluding the large olfactory receptor subfamily, which contains roughly 50% of the class A GPCR sequences.