Published in final edited form as:

J Magn Reson. 2015 April ; 253: 111–118. doi:10.1016/j.jmr.2014.12.014.

Uncovering the triggers for GPCR activation using solid-state NMR spectroscopy

Naoki Kimata^a, Philip J. Reeves^b, and Steven O. Smith^{a,*}

^a Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY 11794-5215, United States

^b School of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, CO4 3SQ, United Kingdom

Abstract

G protein-coupled receptors (GPCRs) span cell membranes with seven transmembrane helices and respond to a diverse array of extracellular signals. Crystal structures of GPCRs have provided key insights into the architecture of these receptors and the role of conserved residues. However, the question of how ligand binding induces the conformational changes that are essential for activation remains largely unanswered. Since the extracellular sequences and structures of GPCRs are not conserved between receptor subfamilies, it is likely that the initial molecular triggers for activation vary depending on the specific type of ligand and receptor. In this article, we describe NMR studies on the rhodopsin subfamily of GPCRs and propose a mechanism for how retinal isomerization switches the receptor to the active conformation. These results suggest a general approach for determining the triggers for activation in other GPCR subfamilies using NMR spectroscopy.

Keywords

Solid-state NMR spectroscopy; G protein-coupled receptor; Magic angle spinning

1. Introduction

G protein-coupled receptors (GPCRs) have a common 7 trans-membrane (TM) helix structure and when activated catalyze the exchange of GTP for GDP in intracellular heterotrimeric G proteins [1]. One of the intriguing questions surrounding GPCRs is how these receptors are able to respond to signals ranging from small molecule odorants to protein hormones. The answer to this question appears to be that the activation mechanism(s) have both common and ligand-specific components. The common element of GPCR activation involves an outward rotation of a single TM helix (H6) upon ligand binding (Fig. 1). This motion exposes the G-protein binding site on the intracellular surface of the receptor. Different subfamilies of receptors (and their associated ligands) seem to

have simply evolved different mechanisms to trigger (or release restrictions on) the outward rotation of H6.

Nevertheless, despite intensive research on GPCRs, the molecular mechanisms that GPCRs use to trigger activation have remained elusive. In this article, we first introduce rhodopsin, the receptor for vision in dim-light, as a model GPCR. Rhodopsin has often been considered an exception within GPCRs since the receptor is light-activated by a covalently attached chromophore, rather than by binding of a diffusible ligand. However, comparisons of the sequences and structures of the light-activated and the ligand-activated GPCRs show that they have conserved structural and functional elements. We describe NMR studies that reveal how rhodopsin is activated by light-induced isomerization of its retinal chromophore and how these studies provide a general approach for determining the activation triggers in the ligand-activated receptors.

2. Rhodopsin as a model GPCR

Rhodopsin basically functions as an on–off switch [4]. Light energy is used to drive the protein from an inactive to an active conformation. All visual receptors from humans to squid contain the 11-*cis* isomer of retinal covalently bound within the 7-TM helix bundle (Fig. 1). In pharmacological terms, the 11-*cis* retinal chromophore acts as an inverse agonist and when bound to the receptor it reduces basal activity to very low levels [5]. Specific molecular interactions lock this light-activated receptor into an inactive conformation in the dark, reducing thermal “noise”. Upon light absorption, the retinal isomerizes rapidly (within 200 femto-seconds) to the all-*trans* configuration, which now functions as the agonist for activation. This isomerization occurs within the tightly packed interior of the protein and results in large steric clashes before the protein relaxes thermally through a series of spectrally distinct intermediates. The final intermediate before the all-*trans* retinal dissociates from the receptor is metarhodopsin II (Meta II), which corresponds to the active state of the receptor. Like rhodopsin, Meta II is stabilized by specific contacts that maintain the receptor in the open, active conformation needed for G-protein activation. As a result, rhodopsin can be thought of as a ligand-activated receptor in which the retinal chromophore performs a dual role: in the dark it is a covalently attached inverse agonist and upon absorption of light it is rapidly photo-converted to a potent agonist.

3. The significance of residue conservation

The visual receptors, including rhodopsin and the cone receptors for color vision, comprise a subfamily within the largest of six families or classes of GPCRs. These receptors are designated as Class A (or Family A) GPCRs and group together by sequence conservation. A second argument for considering rhodopsin as a model GPCR is that it contains most of the residues that are highly conserved across the large Class A family. Understanding the roles of these conserved residues is an important first step in describing the activation mechanism of any GPCR. There are three levels of conservation to be considered. The first level of conservation corresponds to the ~20 signature residues that have high sequence identity across the entire Class A GPCR family (Fig. 2). These residues are often grouped

into structural and functional micro-domains that appear to mediate a common conformational switch involved in receptor activation [6,7].

A second level of conservation corresponds to residues that are highly conserved when considered as a group of chemically similar amino acids. We have previously identified the group of small and weakly polar residues (Ala, Gly, Ser, Cys and Thr) as key determinants in helix–helix interactions [8,9]. The third level of conservation corresponds to residues that are highly conserved within a receptor subfamily. For example, Lys296 is strictly conserved in the visual receptor subfamily as it is the residue that forms a covalent protonated Schiff base linkage to the retinal chromophore.

Most of the highly conserved residues in the Class A GPCR family are located in the TM helices. The “signature” amino acids with sequence identities of >70% have been identified early on as playing important roles in GPCR activation. The conserved prolines on H5, H6 and H7, along with the (E/D)RY and NPxxY motifs, have been extensively studied. Several hydrophobic residues (e.g. Leu79 and Trp161) are highly conserved, but less well studied. Their function becomes clear when described in the context of the group conserved residues and the conformational changes that occur upon activation.

Fig. 3A presents a cross section through the middle of the TM region of rhodopsin illustrating the conserved packing core. The core is composed of both signature (red) and group conserved (blue) amino acids. The most highly conserved residue in the Class A GPCRs is Asn55. Close analysis shows that the amine NH₂ side chain of Asn55 is hydrogen bonded to the backbone carbonyls of Gly51 on TM helix H1 and Ala299 on H7, both group conserved amino acids. Asp83 is part of the highly conserved LAXAD sequence on TM helix H2. Leu79, Ala82 and Asp83 are signature residues, while Ala80 is group conserved. The very high group conservation of Ala80 (97%) allows close packing of H1–H2 and the formation of a specific Asn55–Asp83 hydrogen bonding contact. Ala82 mediates the packing of helix H2 with helices H3 (Ile123) and H4 (Trp161). Together, Ala82 and Ile123 form a pocket that allows hydrogen bonding of Trp161 (indole NH) to Asn78, thereby bridging H2 and H4.

Fig. 3B shows the conformational changes in the TM core upon activation. Several core packing interactions change upon activation. Perhaps the most important are the packing interactions of Met257 and Phe261. These residues are on TM H6 at the level of the TM core and separated by one helical turn. (The TM core is a cross section of largely conserved residues spanning approximately two helical turns.) Met257 packs within the TM core, and its side chain moves down toward Arg135 upon activation (Fig. 4). The notch between Leu128 and Asn302, where Met257 packs in rhodopsin, closes up in Meta II as H6 pivots outward. Mutation of Met257 in the TM core can convert the rhodopsin into a ligand-activated receptor, in which the ligand is exogenously added all-*trans* retinal [10]. Most substitutions of Met257 exhibit low, but measurable, activity without bound retinal. These receptors are locked off by covalent attachment of the “inverse agonist” 11-*cis* retinal, but exhibit activities comparable to the light activated receptor upon binding the “agonist” all-*trans* retinal.

Phe261 is the second residue on H6 that packs in the TM core (above Met257). This position in the Class A GPCRs has a conservation of 75% when the olfactory receptors are not included. The olfactory receptors do not contain the conserved CWxP sequence on helix H6 or an aromatic residue at the position of Phe261. Phe261 shifts toward Pro215 in Meta II. A similar motion is observed in the β_2 -adrenergic receptor for Phe282, which occupies the same position relative to the conserved CWxP sequence [7,11]. The role of this residue as a potential lever in the motion of H6 is highlighted by early studies involving mutation of Gly121, one of the rhodopsin subfamily conserved residues [12]. Mutation of Gly121 to larger residues results in dark activation of rhodopsin [12]. This steric trigger is reversed by the F261A mutation, but not by mutation of Trp265, one helix turn above Phe261 [13]. Below we describe solid-state NMR studies that provide support for Phe261 as a lever in rhodopsin activation through direct interaction with the retinal β -ionone ring.

The outward rotation of H6 is associated with the inward rotation of two highly conserved tyrosine residues on the adjacent helices H5 and H7: Tyr223 and Tyr306, respectively. Upon activation, these tyrosines disrupt the Arg135-Glu247 “ionic lock” that tethers H6 to H3 on the intracellular side of rhodopsin in the inactive state. Tyr223 has a sequence identity of 92% in Class A GPCRs, while Tyr306 (which is part of a conserved NPxxY sequence) has an identity of 96%. Arg135 (97% identity) is part of a conserved ERY sequence at the end of H3. Fig. 4 shows the structure of the Arg135-Glu247 “ionic lock” on the cytoplasmic side of the receptor. Both Glu134 and Glu247 shift away from Arg135 as Tyr223 and Tyr306 rotate inward. Glu134 becomes protonated, while Arg135 is stabilized by hydrogen bonding interactions with these tyrosines.

4. NMR provides a complementary approach to X-ray crystallography

Rhodopsin was the first GPCR whose crystal structure was determined to high resolution [14]. The structure confirmed the seven-helix architecture and revealed the location of amino acids that are highly conserved across the large Class A GPCR family. In the past eight years a number of high-resolution crystal structures of Class A GPCRs have been determined for visual pigments [2,3], amine [15,16], chemokine [17], mAChR [18,19], opioid [20], lipid [21] and δ -subfamilies of receptors [22], the latter including the olfactory receptors. The basic structural elements present in these structures are similar to those observed in rhodopsin. Comparison of rhodopsin with the ligand activated GPCRs revealed that the largest structural diversity occurs in the N-terminus, the extracellular loops and the intracellular loops. For example, on the extra-cellular side of rhodopsin, the second extracellular loop (EL2) is wedged between the TM helices and serves as plug over the retinal-binding site. Thus far, this is the only GPCR showing this structural element.

In contrast to the relatively large number of structures of inactive GPCRs, there are so far few active-state GPCR crystal structures. Active-state crystal structures of ligand-activated receptors that exhibit a large outward motion of H6 have been determined for the β_2 -adrenergic receptor with either a nanobody or the full length G protein bound to the intracellular surface [23,24]. In the presence of agonist alone, the structural changes in the ligand-activated GPCRs are more modest [25,26]. These receptors have relatively small barriers to activation in contrast to rhodopsin where light energy is needed to overcome the

large thermal barrier to activation. In most GPCRs, it appears that multiple receptor conformations can be populated, which provides versatility in signaling and regulation [27].

Agonist-bound structures are available for the A_{2A} adenosine receptor [25,28], the β_1 adrenergic receptor (β_1 AR) [26,29], the β_2 adrenergic receptor (β_2 AR) [11] and the β_2 AR in complex with its cognate G-protein (Gs) [24]. In these structures, the activating ligand binds roughly in the same location on the extracellular side of the TM helix bundle as the retinal chromophore in rhodopsin. The largest change in these structures, as compared to the corresponding inactive conformation, is the displacement of TM helix H6. Agonist-induced conformational changes in other regions of these receptors appear to be minimal, which has left open the question as to how ligand binding triggers activation [30].

In the past few years, several crystal structures have been reported on the active Meta II state of rhodopsin [31–33]. Two structures have been reported on rhodopsin containing mutations (E113Q and M257Y) that lead to constitutive activation [50,51] and a third structure was obtained by diffusing all-*trans* retinal into crystals of opsin to generate a Meta II-like complex. In the E113Q opsin structure, the all-*trans* retinal was not covalently attached (and consequently does not accurately reflect the active conformation). In the Meta II structures obtained with the M257Y mutant and with opsin, the orientation of the retinal is different than the orientation proposed on the basis of solid-state NMR measurements [34,35]. These differences have again raised the question as to how retinal isomerization (or ligand-binding) triggers receptor activation. In the sections below we outline the NMR approach for addressing this question and propose a mechanism that couples retinal isomerization to motion of TM helix H6.

5. A general approach for determining the triggers for GPCR activation

In magic angle spinning (MAS) NMR studies of GPCRs, structural information is obtained through measurements of chemical shift and dipolar couplings. MAS NMR methods do not yet yield complete 3D protein structures of GPCRs due to the expense of uniform ¹³C, ¹⁵N-labeling of proteins using eukaryotic cell lines. Rather, selective labeling of specific amino acid types or different ligands allows one to probe the finer structural features of the ligand or protein that are of interest. The chemical shift of a particular ¹³C or ¹⁵N nucleus depends on the type of chemical group in which it occurs and is modulated by the local structure and environment of that group. Information on secondary structure and hydrogen bonding interactions can be extracted from chemical shift values. Dipolar couplings report on internuclear distances. One major advantage of these methods is that the receptor structure can be probed in a native membrane environment using the native protein sequence or site directed mutants, and low temperature provides a way to trap and stabilize reactive intermediates.

Given their limited bioavailability and inherent instability, obtaining GPCRs in sufficient quantity for structural studies is challenging. The two most widely used heterologous expression systems for large-scale production of functional GPCRs are baculovirus-infected Sf9 insect cells and HEK293S cells. These eukaryotic systems allow for post-*translational* modifications, which are often required for proper receptor folding and function. Recent

progress on cell-free expression has also led to the production of *functional* GPCRs [36]. There are several advantages of heterologous expression of GPCRs in HEK293S cells over Sf9 cells and other systems for the expression of GPCRs. First, these cells generate the largest yield of functional GPCRs. For example, several milligrams of rhodopsin can be extracted from one-liter growths of HEK293S cells, and on the basis of UV/Vis spectroscopy and G protein activation, the receptor is indistinguishable from rhodopsin purified from rod cell outer segments [37]. Second, these cells can be grown to high density in suspension culture using defined media that is specifically formulated for incorporation of ^{13}C and ^{15}N labeled amino acids.

There are two rounds of selection required to obtain cells expressing a GPCR of interest using the HEK293S cell line (ATCC No. CRL1573). The specific methods have been described in detail in the original publications on this cell line adapted for suspension growth [38,39]. The selection process results in stable cell lines expressing the GPCR gene under the control of a tetracycline inducible promoter.

6. NMR studies on how retinal isomerization triggers rhodopsin activation

Fig. 5A shows the 11-*cis* retinal chromophore bound within the interior of rhodopsin on the extracellular side of the TM bundle of helices. The retinal stretches across the binding site and is tightly packed in the binding site cavity. It is attached to Lys296 through a protonated Schiff's base (PSB). Proton transfer from the PSB to Glu113 is an essential element in triggering receptor activation [40]. The retinal β -ionone ring is tightly packed against H5, and is also essential for activation. Truncation of the β -ionone ring results in loss of receptor activity [41,42].

On the extracellular side of the retinal, the second extracellular loop (EL2) forms a plug over the retinal-binding site. The EL2 sequence extends from Trp175 on H4 to Thr198 on H5 and consists of two short β -strands ($\beta 3$ and $\beta 4$). The positions of the β -strands are constrained by a conserved disulfide bond between Cys110 on H3 and Cys187 on $\beta 4$ and a salt bridge between Arg177 and Asp190. A hydrogen-bonded network centered on Glu181 also serves to stabilize the position of EL2. Glu181 is hydrogen bonded to Tyr192 and Tyr268 (on H6), and is connected through water-mediated hydrogen bonds to Ser186 and to Glu113 (on H3), the counterion to the retinal PSB [43]. This stable hydrogen bonded network is thought to be important for keeping the Schiff's base protonated in the dark state of rhodopsin.

On the intracellular side, the retinal is notched over Trp265. Trp265 is part of a conserved CWxP sequence within the extracellular retinal binding region. One helical turn below Trp265 is Phe261, which is a key residue within the TM core (discussed above). One residue above Trp265 is Tyr268, which has a high conservation (98%) within the rhodopsin subfamily. As a result, Trp265 is in a key position; it mediates interactions with the retinal and EL2 through Tyr268, and mediates interactions with Phe261 located in the TM core.

The question is how retinal isomerization triggers rotation of TM 6. The orientation of the retinal is important since both the retinal polyene chain and its associated methyl groups contribute to the ability of the retinal to trigger activation. The C20 methyl group in rhodopsin contacts Trp265 and Tyr268 on H6 in the dark state. When the retinal C20 methyl

group is removed, the photoreaction is slowed [44] and the quantum yield is reduced [45]. Deuterium measurements on the retinal revealed that the C20 methyl group is displaced out of the retinal plane and provided evidence for a ‘pre-twist’ about the C11=C12 double bond [46]. This conformational distortion “primes” the retinal for isomerization [43,47]. The retinal C19 methyl group in rhodopsin is tightly constrained in the retinal binding site and packs against Thr118 (H3), Ile189 (EL2), Tyr191 (EL2) and Tyr268 (H6). Removal of the C19 methyl group results in the complete loss of receptor activity [48], while replacement of the retinal C19 methyl group with an ethyl or propyl group converts the 11-*cis* PSB chromophore from a potent inverse agonist into a partial agonist, with the amount of activity being proportional to the size of the substituent at the C19 position [49]. Below we target these methyl groups and their interactions with the aromatic residues on H6 using specific ^{13}C labeling.

Fig. 5B presents ^{13}C difference spectra between inactive rhodopsin and the active Meta II intermediate. These spectra highlight the amino acids on EL2 that plug the retinal-binding site. There are large chemical shift changes in Ser186, Cys187, Gly188, Ile189 and Tyr191. The chemical shifts of the serine C β —OH and tyrosine C ζ —OH resonances likely reflect changes in C—OH hydrogen bonding. The large downfield shift of Tyr191 corresponds to a strong increase in hydrogen bonding suggesting that there is a rearrangement in the hydrogen-bonding network involving EL2 upon activation.

The ^{13}C difference spectra highlight the residues undergoing changes in orientation or environment upon activation. On the basis of group conserved residues, we had previously proposed that TM helices H1—H4 did not change appreciably upon activation, but rather formed a rigid scaffold enabling motions of H5—H7 [8]. Residues on helices H5—H7, along with the residues on EL2 in contact with the retinal, have exhibited the largest changes in difference spectra. The difference spectra also suggest that the active Meta II intermediate has a single, defined conformation. The number of resonances and their corresponding line widths are similar to those in rhodopsin.

Distance measurements through dipolar couplings have been instrumental in providing insights into the structural transitions occurring upon activation. Fig. 6 presents rows taken out of two-dimensional solid-state NMR spectra illustrating the contacts between the retinal methyl groups (C18, C19 and C20) and the aromatic cluster on H6 (Phe261, Trp265 and Tyr268) in rhodopsin and Meta II. The experiments were run with the retinal ^{13}C -labeled at the C18, C19 or C20 methyl groups and ^{13}C -labels on these aromatic amino acids.

With this limited set of NMR data, one can begin to understand how retinal isomerization is coupled to activation. In Figs. 7 and 8, we highlight the triggers for activation from the standpoint of the C18, C19 and C20 retinal methyl groups, and the conserved aromatic residues (Phe261, Trp265 and Tyr268) on H6. Fig. 7 shows the position of Phe261 and C18. The 11-*cis* retinal chromophore is longitudinally restricted within its binding pocket [51] and isomerization results in strong steric interactions with surrounding residues. Phe261 is in contact with the β -ionone ring and Trp265 occupies the space between the β -ionone ring and the Lys296 side chain. Mutation of Phe261 results in a 60% loss of signaling activity [13]. The NMR data show a strong increase in the intensity of the cross peak between Phe261 and

the C18 methyl group of the ionone ring, consistent with motion of the ring toward H5 and the loss of activity if either Phe261 is mutated or the β -ionone ring is truncated. In contrast, the Meta II crystal structures place the retinal much higher in the binding site and in an orientation where the C18 methyl group is >10 Å from Phe261.

Fig. 8 shows the positions of the C19 and C20 methyl groups relative to key residues responsible for activation. Upon light absorption, the retinal isomerizes about the C11=C12 double bond, which is located between the C19 and C20 methyl groups. The C20 methyl group very likely rotates in a clockwise fashion (when viewed from the Lys296 end of the retinal). This motion moves the Schiff base NH proton away from Glu113, its counterion in the dark state, and triggers deprotonation of the protonated Schiff base. Following isomerization the C20 methyl group is in close proximity to Tyr268 as reflected in the Tyr-C20 contact observed in Fig. 6c.

The C19 methyl group rotates in a counterclockwise direction toward Tyr191. We propose that rotation of the C19 methyl group disrupts the hydrogen-bonding network between Tyr191, Tyr268 and Glu181, and that the rearrangement of this network is essential for Schiff base deprotonation. Tyr191 is packed against the extracellular end of H6. Motion of this tyrosine toward the Glu181 and the Schiff base would allow the extracellular end of H6 to pivot. We propose that this motion facilitates the outward rotation of the intracellular end of H6. This pivoting motion is captured in the global toggle switch mechanism proposed by Schwartz and coworkers on the basis of mutational data [52,53], but is not observed in the crystal structures of Meta II. Motion of H6 and rotation of Trp265 places the aromatic ring of this conserved tryptophan into close proximity of the C19 methyl group as reflected in the C19-Trp contact observed in Fig. 6B.

The picture that emerges from this analysis of activation is that there is not a simple molecular switch. When the first crystal structures emerged for rhodopsin, it was unclear how retinal isomerization resulted in TM helix motions. NMR has provided strong evidence for a possible mechanism. The subtle changes in chemical shift and dipolar couplings that we can measure explain how the steric and electrostatic interactions of the retinal within its binding pocket release constraints holding H6 in an inactive conformation (e.g. Tyr191) and drive this helix into an activation conformation (e.g. contacts between the retinal ionone ring and Phe261).

Similar measurements in the ligand-activated GPCRs can provide clues to understanding their ligand-specific triggers. These GPCRs typically exhibit basal activity and can be inhibited by binding inverse agonists or activated by binding partial or full agonists. The first step would be to obtain NMR difference spectra between receptors with a bound inverse agonist and a bound full agonist, corresponding to the largest difference in receptor activity. The approach is to target residues that are conserved within a given subfamily of receptors and to make use of crystal structures to guide the design and interpretation of experiments. Measurements of internuclear dipolar couplings provide constraints on the structures of the inactive and active conformations, and the conformational change that occurs upon activation.

Acknowledgment

This work was supported by a Grant from the National Institutes of Health (RO1-GM41412) to SOS.

References

1. Rosenbaum DM, Rasmussen SGF, Kobilka BK. The structure and function of G-protein-coupled receptors. *Nature*. 2009; 459:356–363. [PubMed: 19458711]
2. Park JH, Scheerer P, Hofmann KP, Choe HW, Ernst OP. Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature*. 2008; 454:183–187. [PubMed: 18563085]
3. Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP, Ernst OP. Crystal structure of opsin in its G-protein-interacting conformation. *Nature*. 2008; 455:497–502. [PubMed: 18818650]
4. Smith SO. Structure and activation of the visual pigment rhodopsin. *Ann. Rev. Biophys.* 2010; 39:309–328. [PubMed: 20192770]
5. Vogel R, Siebert F. Conformations of the active and inactive states of opsin. *J. Biol. Chem.* 2001; 276:38487–38493. [PubMed: 11502747]
6. Ahuja S, Smith SO. Multiple switches in G protein-coupled receptor activation. *Trends Pharmacol. Sci.* 2009; 30:494–502. [PubMed: 19732972]
7. Valentin-Hansen L, Holst B, Frimurer TM, Schwartz TW. PheVI:09 (Phe6.44) as a sliding microswitch in seven-transmembrane (7TM) G protein-coupled receptor activation. *J. Biol. Chem.* 2012; 287:43516–43526. [PubMed: 23135271]
8. Liu W, Eilers M, Patel AB, Smith SO. Helix packing moments reveal diversity and conservation in membrane protein structure. *J. Mol. Biol.* 2004; 337:713–729. [PubMed: 15019789]
9. Eilers M, Patel AB, Liu W, Smith SO. Comparison of helix interactions in membrane and soluble a-bundle proteins. *Biophys. J.* 2002; 82:2720–2736. [PubMed: 11964258]
10. Han M, Smith SO, Sakmar TP. Constitutive activation of opsin by mutation of methionine 257 on transmembrane helix 6. *Biochemistry*. 1998; 37:8253–8261. [PubMed: 9609722]
11. Rosenbaum DM, Zhang C, Lyons JA, Holl R, Aragao D, Arlow DH, Rasmussen SGF, Choi HJ, DeVree BT, Sunahara RK, Chae PS, Gellman SH, Dror RO, Shaw DE, Weis WI, Caffrey M, Gmeiner P, Kobilka BK. Structure and function of an irreversible agonist-b₂ adrenoceptor complex. *Nature*. 2011; 469:236–240. [PubMed: 21228876]
12. Han M, Lin SW, Smith SO, Sakmar TP. The effects of amino acid replacements of glycine 121 on transmembrane helix 3 of rhodopsin. *J. Biol. Chem.* 1996; 271:32330–32336. [PubMed: 8943295]
13. Han M, Lin SW, Minkova M, Smith SO, Sakmar TP. Functional interaction of transmembrane helices 3 and 6 in rhodopsin –replacement of phenylalanine 261 by alanine causes reversion of phenotype of a glycine 121 replacement mutant. *J. Biol. Chem.* 1996; 271:32337–32342. [PubMed: 8943296]
14. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M. Crystal structure of rhodopsin: A G protein-coupled receptor. *Science*. 2000; 289:739–745. [PubMed: 10926528]
15. Rasmussen SGF, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VRP, Sanishvili R, Fischetti RF, Schertler GFX, Weis WI, Kobilka BK. Crystal structure of the human b₂ adrenergic G-protein-coupled receptor. *Nature*. 2007; 450:383–387. [PubMed: 17952055]
16. Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SGF, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, Stevens RC. High-resolution crystal structure of an engineered human b₂-adrenergic G protein-coupled receptor. *Science*. 2007; 318:1258–1265. [PubMed: 17962520]
17. Wu B, Chien EY, Mol CD, Fenalti G, Liu W, Katritch V, Abagyan R, Brooun A, Wells P, Bi FC, Hamel DJ, Kuhn P, Handel TM, Cherezov V, Stevens RC. Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science*. 2010; 330:1066–1071. [PubMed: 20929726]

18. Kruse AC, Hu J, Pan AC, Arlow DH, Rosenbaum DM, Rosemond E, Green HF, Liu T, Chae PS, Dror RO, Shaw DE, Weis WI, Wess J, Kobilka BK. Structure and dynamics of the M3 muscarinic acetylcholine receptor. *Nature*. 2012; 482:552–556. [PubMed: 22358844]
19. Haga K, Kruse AC, Asada H, Yurugi-Kobayashi T, Shiroishi M, Zhang C, Weis WI, Okada T, Kobilka BK, Haga T, Kobayashi T. Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. *Nature*. 2012; 482:547–551. [PubMed: 22278061]
20. Wu H, Wacker D, Mileni M, Katritch V, Han GW, Vardy E, Liu W, Thompson AA, Huang X-P, Carroll FI, Mascarella SW, Westkaemper RB, Mosier PD, Roth BL, Cherezov V, Stevens RC. Structure of the human μ -opioid receptor in complex with JDTic. *Nature*. 2012; 485:327–332. [PubMed: 22437504]
21. Hanson MA, Roth CB, Jo E, Griffith MT, Scott FL, Reinhart G, Desale H, Clemons B, Cahalan SM, Schuerer SC, Sanna MG, Han GW, Kuhn P, Rosen H, Stevens RC. Crystal structure of a lipid G protein-coupled receptor. *Science*. 2012; 335:851–855. [PubMed: 22344443]
22. Zhang C, Srinivasan Y, Arlow DH, Fung JJ, Palmer D, Zheng Y, Green HF, Pandey A, Dror RO, Shaw DE, Weis WI, Coughlin SR, Kobilka BK. High-resolution crystal structure of human protease-activated receptor 1. *Nature*. 2012; 492:387–392. [PubMed: 23222541]
23. Rasmussen SGF, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, DeVree BT, Rosenbaum DM, Thian FS, Kobilka TS, Schnapp A, Konetzki I, Sunahara RK, Gellman SH, Pautsch A, Steyaert J, Weis WI, Kobilka BK. Structure of a nanobody-stabilized active state of the β_2 adrenoceptor. *Nature*. 2011; 469:175–180. [PubMed: 21228869]
24. Rasmussen SGF, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, Mathiesen JM, Shah STA, Lyons JA, Caffrey M, Gellman SH, Steyaert J, Skinotis G, Weis WI, Sunahara RK, Kobilka BK. Crystal structure of the β_2 adrenergic receptor-Gs protein complex. *Nature*. 2011; 477:549–555. [PubMed: 21772288]
25. Xu F, Wu HX, Katritch V, Han GW, Jacobson KA, Gao ZG, Cherezov V, Stevens RC. Structure of an agonist-bound human A_{2A} adenosine receptor. *Science*. 2011; 332:322–327. [PubMed: 21393508]
26. Warne T, Edwards PC, Leslie AGW, Tate CG. Crystal structures of a stabilized $\beta(1)$ -adrenoceptor bound to the biased agonists bucindolol and carvedilol. *Structure*. 2012; 20:841–849. [PubMed: 22579251]
27. Kobilka BK, Deupi X. Conformational complexity of G-protein-coupled receptors. *Trends Pharmacol. Sci.* 2007; 28:397–406. [PubMed: 17629961]
28. Lebon G, Warne T, Edwards PC, Bennett K, Langmead CJ, Leslie AGW, Tate CG. Agonist-bound adenosine A_{2A} receptor structures reveal common features of GPCR activation. *Nature*. 2011; 474:521–525. [PubMed: 21593763]
29. Warne T, Moukhametzianov R, Baker JG, Nehmé R, Edwards PC, Leslie AGW, Schertler GFX, Tate CG. The structural basis for agonist and partial agonist action on a β_1 -adrenergic receptor. *Nature*. 2011; 469:241–244. [PubMed: 21228877]
30. Deupi X, Standfuss J. Structural insights into agonist-induced activation of G-protein-coupled receptors. *Curr. Opin. Struct. Biol.* 2011; 21:541–551. [PubMed: 21723721]
31. Standfuss J, Edwards PC, D'Antona A, Fransen M, Xie G, Oprian DD, Schertler GF. The structural basis of agonist-induced activation in constitutively active rhodopsin. *Nature*. 2011; 471:656–660. [PubMed: 21389983]
32. Deupi X, Edwards P, Singhal A, Nickle B, Oprian D, Schertler G, Standfuss J. Stabilized G protein binding site in the structure of constitutively active metarhodopsin-II. *Proc. Natl. Acad. Sci. U.S.A.* 2012; 109:119–124. [PubMed: 22198838]
33. Choe HW, Kim YJ, Park JH, Morizumi T, Pai EF, Krauss N, Hofmann KP, Scheerer P, Ernst OP. Crystal structure of metarhodopsin II. *Nature*. 2011; 471:651–655. [PubMed: 21389988]
34. Ahuja S, Crocker E, Eilers M, Hornak V, Hirshfeld A, Ziliox M, Syrett N, Reeves PJ, Khorana HG, Sheves M, Smith SO. Location of the retinal chromophore in the activated state of rhodopsin. *J. Biol. Chem.* 2009; 284:10190–10201. [PubMed: 19176531]
35. Ahuja S, Hornak V, Yan ECY, Syrett N, Goncalves JA, Hirshfeld A, Ziliox M, Sakmar TP, Sheves M, Reeves PJ, Smith SO, Eilers M. Helix movement is coupled to displacement of the second

- extracellular loop in rhodopsin activation. *Nat. Struct. Mol. Biol.* 2009; 16:168–175. [PubMed: 19182802]
36. Klammt C, Schwarz D, Eifler N, Engel A, Piehler J, Haase W, Hahn S, Dötsch V, Bernhard F. Cell-free production of G protein-coupled receptors for functional and structural studies. *J. Struct. Biol.* 2007; 158:482–493. [PubMed: 17350285]
 37. Reeves PJ, Thurmond RL, Khorana HG. Structure and function in rhodopsin: High level expression of a synthetic bovine opsin gene and its mutants in stable mammalian cell lines. *Proc. Natl. Acad. Sci. U.S.A.* 1996; 93:11487–11492. [PubMed: 8876162]
 38. Reeves PJ, Hwa J, Khorana HG. Structure and function in rhodopsin: Kinetic studies of retinal binding to purified opsin mutants in defined phospholipid-detergent mixtures serve as probes of the retinal binding pocket. *Proc. Natl. Acad. Sci. U.S.A.* 1999; 96:1927–1931. [PubMed: 10051571]
 39. Reeves PJ, Kim JM, Khorana HG. Structure and function in rhodopsin: a tetracycline-inducible system in stable mammalian cell lines for high-level expression of opsin mutants. *Proc. Natl. Acad. Sci. U.S.A.* 2002; 99:13413–13418. [PubMed: 12370422]
 40. Mahalingam M, Martinez-Mayorga K, Brown MF, Vogel R. Two protonation switches control rhodopsin activation in membranes. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:17795–17800. [PubMed: 18997017]
 41. Jäger F, Jäger S, Kräutle O, Friedman N, Sheves M, Hofmann KP, Siebert F. Interactions of the b-ionone ring with the protein in the visual pigment rhodopsin control the activation mechanism. An FTIR and fluorescence study on artificial vertebrate rhodopsins. *Biochemistry.* 1994; 33:7389–7397. [PubMed: 8003504]
 42. Vogel R, Siebert F, Lüdeke S, Hirshfeld A, Sheves M. Agonists and partial agonists of rhodopsin: retinals with ring modifications. *Biochemistry.* 2005; 44:11684–11699. [PubMed: 16128569]
 43. Okada T, Sugihara M, Bondar AN, Elstner M, Entel P, Buss V. The retinal conformation and its environment in rhodopsin in light of a new 2.2 Å crystal structure. *J. Mol. Biol.* 2004; 342:571–583. [PubMed: 15327956]
 44. Wang Q, Kochendoerfer GG, Schoenlein RW, Verdegem PJE, Lugtenburg J, Mathies RA, Shank CV. Femtosecond spectroscopy of a 13-demethylrhodopsin visual pigment analogue: the role of nonbonded interactions in the isomerization process. *J. Phys. Chem.* 1996; 100:17388–17394.
 45. Kochendoerfer GG, Verdegem PJE, van der Hoef I, Lugtenburg J, Mathies RA. Retinal analog study of the role of steric interactions in the excited state isomerization dynamics of rhodopsin. *Biochemistry.* 1996; 35:16230–16240. [PubMed: 8973196]
 46. Salgado GFJ, Struts AV, Tanaka K, Krane S, Nakanishi K, Brown MF. Solid-state ^2H NMR structure of retinal in metarhodopsin I. *J. Am. Chem. Soc.* 2006; 128:11067–11071. [PubMed: 16925423]
 47. Struts AV, Salgado GFJ, Tanaka K, Krane S, Nakanishi K, Brown MF. Structural analysis and dynamics of retinal chromophore in dark and Meta I states of rhodopsin from ^2H NMR of aligned membranes. *J. Mol. Biol.* 2007; 372:50–66. [PubMed: 17640664]
 48. Corson DW, Cornwall MC, MacNichol EF, Tsang S, Derguini F, Crouch RK, Nakanishi K. Relief of opsin desensitization and prolonged excitation of rod photoreceptors by 9-desmethylretinal. *Proc. Natl. Acad. Sci. U.S.A.* 1994; 91:6958–6962. [PubMed: 8041729]
 49. Han M, Groesbeek M, Sakmar TP, Smith SO. The C9 methyl group of retinal interacts with glycine-121 in rhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* 1997; 94:13442–13447. [PubMed: 9391044]
 50. Takegoshi K, Nakamura S, Terao T. ^{13}C – ^1H dipolar-assisted rotational resonance in magic-angle spinning NMR. *Chem. Phys. Lett.* 2001; 344:631–637.
 51. Matsumoto H, Yoshizawa T. Recognition of opsin to longitudinal length of retinal isomers in formation of rhodopsin. *Vision. Res.* 1978; 18:607–609. [PubMed: 664347]
 52. Elling CE, Frimurer TM, Gerlach LO, Jorgensen R, Holst B, Schwartz TW. Metal ion site engineering indicates a global toggle switch model for seven-transmembrane receptor activation. *J. Biol. Chem.* 2006; 281:17337–17346. [PubMed: 16567806]

53. Schwartz TW, Frimurer TM, Holst B, Rosenkilde MM, Elling CE. Molecular mechanism of 7TM receptor activation –a global toggle switch model. *Annu. Rev. Pharmacol. Toxicol.* 2006; 46:481–519. [PubMed: 16402913]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1.

Structure and activation of rhodopsin. GPCRs consist of a bundle of seven transmembrane helices. In panel (A), the crystal structure is shown of the dark, inactive state of rhodopsin. The 11-*cis* retinal chromophore (red spheres) is bound within the interior of the 7-TM helix bundle on the extracellular side of the receptor. Light induced isomerization of the retinal results in the outward rotation of TM helix H6. The inactive (gray) and active (purple) structures are superimposed in panel (B). The effect of helix H6 motion is to open up a cavity for G-protein binding on the intracellular side of the receptor (C). To illustrate the position of the cavity, in panel (C) we show the C-terminus of the α -subunit of the G-protein (blue helix) that was co-crystallized with the receptor in the active state [2,3]. The activation mechanism involves three regions of the receptor (the extracellular ligand binding region, the TM core, and the intracellular tyrosine switch) that are discussed in the article below. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Schematic diagram of the seven TM helices of rhodopsin highlighting the conserved residues and the three key regions of the receptor. The different levels of conservation are shown. The signature residues (red) are amino acids that have high sequence identity (>70%) across the class A GPCRs. In this case, we have excluded the olfactory receptor subfamily in the analysis since this subfamily does not contain the conserved aromatic residues on H6, which are key to triggering activation in rhodopsin and many of the ligand-activated GPCRs, as described below. The small and polar group conserved residues (blue) are those with a combined conservation of >70%. The residues highlighted in green are those with conservation of >90% across the rhodopsin subfamily. The residues in black are additional residues described in the text. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Packing within the transmembrane core of rhodopsin (A) and Meta II (B). Cross sections are shown from the crystal structures of rhodopsin (PDB ID: 1U19) and Meta II (PDB ID: 3PQR). The signature residues are highlighted in red and the group conserved residues in blue. Met257 (green) has high conservation within the rhodopsin subfamily. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Intracellular tyrosine switch in rhodopsin (A) and Meta II (B). In the inactive state Tyr223 is oriented away from the helix bundle and Tyr306 is hydrogen bonded to Asn73, a rhodopsin subfamily-conserved residue that helps hold rhodopsin in an inactive conformation in the dark. The outward rotation of H6 and breaking of the Arg135-Glu247 interaction is accompanied by the inward rotation of these two tyrosines. Tyr223 packs against the side chain of Ala132 in the active state. Ala132 is one of the most highly group conserved residues and helps position Tyr223 in the active conformation. Cross sections are shown from the crystal structures of rhodopsin (PDB ID: 1U19) and Meta II (PDB ID: 3PQR).

Fig. 5. Structure of the retinal binding site (A) and ^{13}C difference spectra of amino acids in EL2 (B). The ^{13}C difference NMR spectra between rhodopsin and Meta II are shown using protein with ^{13}C labels at $^{13}\text{C}\beta$ -cysteine, $^{13}\text{C}\beta$ -serine, $^{13}\text{C}\alpha$ -glycine, $^{13}\text{C}=\text{O}$ isoleucine and $^{13}\text{C}\zeta$ -tyrosine. The residue assignments were obtained by mutation or by measurements of through-space dipolar couplings to assigned near-by residues.

Fig. 6. Retinal-protein contacts in inactive rhodopsin and activated Meta II. The panels present one-dimensional rows extracted from 2D dipolar assisted rotational resonance (DARR) spectra [50]. In (A), the row is taken through the diagonal resonance of the retinal C18 carbon. In this spectrum the C5—C18 cross peak corresponds to a directly bonded interaction. In (B), the row is taken through the diagonal resonance of the Trp aromatic carbons. In (C), the row is taken through the C20 diagonal resonance. The intensities of the cross peaks are related to internuclear separation between the ^{13}C -labeled sites.

Fig. 7.

Triggers associated with rhodopsin activation. (A) View of the 11-*cis* retinal from the H5—H7 side of the protein. This view shows that the 11-*cis* retinal wraps around Trp265 which prevents H6 motion. Retinal isomerization releases this constraint and results in a strong steric contact between the β -ionone ring and Phe261. (B) View of the 11-*cis* retinal from the H4—H5 side of the protein. The H4 and H5 helices have been removed for clarity. The retinal packs between H3 and H6. Phe261 and Trp265 are in close contact with Gly121 on H3. We propose that retinal isomerization drives the β -ionone ring toward Phe261, which serves a lever for the outward rotation of H6.

Fig. 8.

Triggers associated with rhodopsin activation. (A) View of the 11-*cis* retinal chromophore in rhodopsin from the extracellular surface. The hydrogen bonding network is shown between Tyr191, Tyr268 and Glu181, and between Glu113 and the Schiff base proton. (B) View of the 11-*cis* retinal chromophore in relationship to Phe261, Trp265 and Tyr268 on H6. We propose that retinal isomerization drives the Schiff base proton away from Glu113, which facilitates deprotonation. In concert with this motion, there is a shift of Glu181 (along with Tyr191 and Tyr268) toward the Schiff base end of the retinal. This motion releases the packing interaction of Tyr191 with H6 allowing the extracellular end of H6 to pivot.