

THE CRYSTALLOGRAPHIC MODEL OF RHODOPSIN AND ITS USE IN STUDIES OF OTHER G PROTEIN–COUPLED RECEPTORS

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■ **Abstract** G protein–coupled receptors (GPCRs) are integral membrane proteins that respond to environmental signals and initiate signal transduction pathways activating cellular processes. Rhodopsin is a GPCR found in rod cells in retina where it functions as a photopigment. Its molecular structure is known from cryo-electron microscopic and X-ray crystallographic studies, and this has reshaped many structure/function questions important in vision science. In addition, this first GPCR structure has provided a structural template for studies of other GPCRs, including many known drug targets. After presenting an overview of the major structural elements of rhodopsin, recent literature covering the use of the rhodopsin structure in analyzing other GPCRs will be summarized. Use of the rhodopsin structural model to understand the structure and function of other GPCRs provides strong evidence validating the structural model.

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INTRODUCTION

G protein-coupled receptors (GPCRs) are a large group of integral membrane proteins that provide molecular links between extracellular signals and intracellular processes (15, 36, 54, 61, 117, 121). Proteins in this class respond to stimuli such as molecular ligands or light and couple these to internal signal transduction systems involving G proteins. The receptors are sensors in molecular level communication systems connecting external signals with intracellular functions and pathways.

Several hundred GPCRs are found in tissues throughout the human body, where they respond to a range of signals and ligands. For instance, rhodopsin and the cone opsins respond to light, other receptors respond to small molecular signals such as dopamine, histamine, or serotonin, and yet other receptors bind to larger ligands such as angiotensins or chemokines. The roles of these GPCRs in fundamental cellular processes make them important drug targets. A large fraction of the clinically important drugs in use today are targeted toward GPCRs (6).

One of the most widely studied GPCRs is rhodopsin, the photopigment found in the visual system (32, 44, 72, 78, 81, 85, 94, 95, 102). This protein (with its retinal chromophore) is found in the outer segments of retinal rod cells. Absorption of a photon causes the retinal chromophore to change its conformation from *11-cis* to *all-trans*. This is accompanied by conformational changes in the protein that result in a binding site on its cytoplasmic surface for its cognate G protein, transducin (Gt) (30, 43, 71, 90). Once activated, the α subunit of Gt activates a phosphodiesterase that converts cyclic-GMP to GMP. Ion channels gated by cyclic-GMP then close, leading to a hyperpolarized cell that can initiate a nerve signal from the retina to the brain.

Biological, chemical, and physical studies of rhodopsin, both as a vision protein and as a prototypical GPCR, have taken advantage of its high abundance in bovine eyes. Many of the methods used to probe GPCR structure and function were first applied to rhodopsin because of its availability. This also contributed to the efforts leading to a three-dimensional crystal structure determination of rhodopsin (86).

The first part of this article provides an overview of the structural features of ground-state rhodopsin determined using X-ray crystallographic methods. More detailed summaries of the structure and function of rhodopsin are found in References 32 and 95. The second part of this review examines the application of the rhodopsin structural model in studies of other GPCRs.

STRUCTURAL STUDIES OF RHODOPSIN

The seven transmembrane helices in the structural core of GPCRs were first observed in electron microscopic studies of two-dimensional crystals of bovine rhodopsin (97). In 1997, a low-resolution view of the helices was obtained from cryo-electron microscopic studies of two-dimensional crystals of frog rhodopsin (114).

These basic structures, along with the higher-resolution bacteriorhodopsin models (26, 65, 66, 87, 110), supported efforts to computationally model many GPCRs (5, 89). In addition, other biophysical techniques, including NMR (1, 20, 27, 126), spin-label EPR (2, 3), and disulfide formation rates (18, 57), were also used to obtain structural information about these molecules.

Tetsuji Okada, in Palczewski's laboratory, succeeded in obtaining three-dimensional crystals of bovine rhodopsin (80) using protein purified with a protocol he developed (82). One important contribution to his success was his ability to stabilize the protein (and the crystal) by addition of Zn^{2+} in the purification. This is consistent with recent ideas coupling membrane protein stability with successful structural studies (93). Whether the techniques successful for crystallization of rhodopsin can be applied to other GPCRs is an open question.

The crystallographic structure was solved using multi-wavelength anomalous dispersion techniques on a mercury derivative (86). Crystals with high twinning ratios provided diffraction data sufficient to show the helical core of the structure, but not the connecting loops on either end of the molecule. Miyano and coworkers at the SPring8 synchrotron identified a crystal with a low twinning ratio and used it to obtain phases showing the entire molecule.

The structure was refined using standard crystallographic techniques for twinned crystals. Three coordinate sets are currently (June 2002) available from the Protein Data Bank (13). The set with identification code 1F88 is the model reported in the initial structure description (86). Model 1HZX was obtained by further refinement of 1F88 (112). Model 1L9H was obtained by refinement at slightly higher resolution, 2.6 Å (79). Small conformational differences between the models are well within the error estimates for these resolution limits. In the case of 1HZX and 1L9H, various nonprotein components of the structure have been added to the model: zinc, mercury, water, heptane-1,2,3-triol, β -nonyl glucoside, palmitoyl groups, and carbohydrate. These structures were determined at a higher resolution than previously seen for GPCRs, but these are not "high-resolution" structures. There is a serious lack of structural detail available at 2.8 or 2.6 Å resolution. The structural models can be expected to change as higher-resolution data become available.

The residue numbering scheme used in this article combines the generic system of Ballesteros & Weinstein (9) and the residue numbers for the particular receptor being discussed. As an example, consider K7.43[296]. The residue is number 7.43 in the generic numbering system and number 296 in the bovine rhodopsin sequence. In the former, the first digit denotes which helix contains the residue

and the second number is an index locating the residue in the helix. The most conserved residue in each helix is assigned an index of 50, so residue Lys7.43 is located in helix 7 and is 7 (= 50–43) residues before the most conserved residue.

MOLECULAR STRUCTURE OF RHODOPSIN

Overview of the Structure

The crystallographic model of rhodopsin (Figure 1) confirmed many of the previously known or assumed structural features for GPCRs. The seven transmembrane helices are arranged as seen in the cryo-electron microscopy studies, with the same topology as found in bacteriorhodopsin (46).

The N terminus of the protein is on the extracellular side of the membrane. This is also the intradiscal side in the membrane disks found in rod cells in the retina. The helices then traverse the membrane with odd-numbered helices going from the extracellular/intradiscal side to the intracellular/cytoplasmic side when passing along the polypeptide from the N terminus to the C terminus. There are three extracellular loops connecting helices on that side of the membrane. Along with the N-terminal tail, these make up the extracellular surface. Three cytoplasmic loops as well as the C-terminal region form the intracellular surface of the protein.

Cytoplasmic Loops

The temperature factors for the refined model show an interesting trend in that their values are higher toward the cytoplasmic side of the protein. The cytoplasmic surface of the protein is where its Gt will bind. Accordingly, the major conformational shifts initiating the signaling cascade should occur on this face. The structure is consistent with this in that one of the cytoplasmic loops (C-III) and part of the C-terminal tail are not seen in the electron density maps. The structural disorder accounting for this might be either static or dynamic. These parts of the protein appear to be more flexible, a characteristic that could be useful for rearranging a binding site for Gt.

Bent Helices

The transmembrane helices in rhodopsin are not regular α -helices. The helices are bent and contain segments with 3_{10} - or π -helical conformations. Detailed analyses of these distortions (92) have shown that in many of the helices, Pro residues are associated with the bends. The conformational restraints imposed on the backbone atoms of Pro by its side chain ring do not keep this residue from being located in helical regions of Φ - Ψ space. The major effect of Pro on a helical structure is removal of a hydrogen bond due to the lack of a hydrogen atom on the imino nitrogen. Although this destroys the continuity of the hydrogen-bonding pattern for a helix, it is not sufficient to cause a distortion in the helical structure. The

methylene carbons of Pro account for that. In a potential α -helix, the carbonyl oxygen of a residue four positions before the Pro would be its hydrogen-bond acceptor. The δ -methylene of Pro is located where the hydrogen atom of an amino acid would normally be found. This is too close to the carbonyl oxygen, so to accommodate the packing of the atoms, the carbonyl group tilts away from the helical axis. This introduces a distortion in the backbone conformational angles that results in a bending of the entire helix. This type of distortion was pointed out in the past (67, 91, 96, 119), and it and related distortions are found in 6 of the 10 helical bends in rhodopsin.

The bend of helix II is different in that it is associated with an introduction of an extra residue in one turn of the helix. The residues involved in this turn take on backbone conformational angles characteristic of π -helices. This is a rare conformational state for residues found in protein structures, but the presence of an extra residue in this turn of helix is not a resolution artifact.

The turn of π -helix in helix II contains two adjacent Gly residues (G2.56[89] and G2.57[90]). This Gly-Gly pattern is conserved in many GPCRs (49). Gly residues, with no bulky side chains, can take on conformational angles that other residues cannot. However, the reason adjacent Gly residues are associated with an insertion of an extra residue in the helix is not apparent.

The bends in the helices, and the twists associated with them, might be hinge-points for relevant conformational changes associated with GPCR activation (119). The hydrogen-bond patterns are already broken at the bends, so further bending of the helices should have minor enthalpic effects. Several investigators have suggested that the helical bending has functional significance in these molecules, so it will be interesting to see if that idea is supported by structural studies of complexes of the receptors and their G proteins.

Chromophore Conformation and Binding Site

The importance of the conformational change in the chromophore has made it the object of extensive biophysical studies (59, 70). In the ground-state inactive form of the protein, the polyene segment of the chromophore is in the *11-cis* conformation. NMR and cross-linking studies (16, 17, 104, 106, 111) indicate that the β -ionone ring is oriented to give the *6-s-cis* conformer. This is consistent with the X-ray diffraction pattern.

The interactions of the chromophore with the surrounding protein residues are of interest because they influence the absorption spectrum of the protein/chromophore complex. In addition, they might serve as a model of ligand binding sites in other GPCRs. Hydrophobic groups largely cover the surface of the retinal binding cavity. The β -ionone ring is packed between two Phe residues (F5.47[212] and F6.44[261]), while the side chain of W6.48[265] is located in the center of the site with the chromophore bent around it in the *11-cis* conformation. Although the chromophore is completely buried inside the protein with no accessibility to the aqueous or membrane environment, there are several polar or charged protein

side chains nearby. Burial of polar groups within the protein is thermodynamically costly, so their presence near the retinal is likely due to important structural or functional reasons. T3.33[118] and Y5.61[268] are located near the polyene tail, but the major charged group associated with the retinal is E3.28[113]. This residue is located near the Schiff base linkage of the chromophore with K7.43[296] and likely serves as a counterion when the Schiff base is protonated.

Major candidates for comparative structure/function studies with rhodopsin are the cone opsins, the related molecules in cone cells responsible for wavelength discrimination and color vision (102, 108). Description of those comparisons lies outside the scope of this review.

Activation and Interactions with G Proteins

After the quick *cis-trans* isomerization of the chromophore, rhodopsin passes through a set of conformational states that can be characterized spectroscopically (102). One of these photostates, Meta II, is the form of the protein capable of activating Gt. The α subunit of Gt then extends the signal transduction cascade. The structural changes leading to Meta II are believed to be similar in the activation of most GPCRs.

In the case of rhodopsin, structural information concerning activation is limited because we have a static, time-averaged view of only the initial state in the crystals. However, investigators have used mutagenesis to introduce Cys residues into rhodopsin for use in disulfide formation, accessibility, and spin-label experiments probing the structure of the activated and ground-state molecules (2, 3, 18, 57, 72, 78). These studies and others have shown which transmembrane helices must move during activation, and by how much. The interresidue distances obtained using these techniques are affected by chemical modifications and perturbations of the protein structure, but the experiments provide the best information currently available about the activation process, albeit without a lot of atomic level detail.

Several papers have appeared illustrating and summarizing the major structural principles connected with GPCR activation (3, 32, 43, 71, 78). The response of GPCRs to a signal is to expand and open up their structures on the cytoplasmic side of the membrane. This is accomplished by movement of the cytoplasmic ends of helices II, VI, and VII to create a binding crevice across the cytoplasmic surface of the protein. No reports are yet available describing detailed models of an activated receptor or a receptor/G protein complex.

MODELING OF OTHER GPCRS

The molecular model of bovine rhodopsin provides a useful structural framework for understanding the large amounts of structural and functional data compiled for the vision photopigments. The 2.8 Å resolution structural model has also been important for researchers focusing on the chemistry and biology of GPCRs. Because three-dimensional structures for membrane proteins are not readily available,

computational modeling of GPCRs has been an active research area (5, 35, 47, 89, 118). Several important issues complicate modeling of GPCRs based on the model of rhodopsin. One is the low sequence identity between rhodopsin and other receptors (116, 118). Alignment of the amino acid sequences for these proteins is also complicated by variation in the lengths of the interhelical loops (84). In addition, the retinal binding pocket is completely buried in the protein and may make it an unsuitable model for the binding of ligands to most other GPCRs (69). If the dynamics of the protein permit occasional access to the binding site from the aqueous or membrane environment, this might not be an issue. Finally, the currently available structure is for the inactive form of the receptor, and this should be remembered if activated receptors are being modeled.

The next sections summarize how the three-dimensional crystal structure of bovine rhodopsin has been used to understand functional or structural characteristics of other GPCRs. Some of the studies cited record use of the rhodopsin model itself for understanding a particular GPCR while others describe homology models built to explain chemical and biochemical properties of the receptor of interest. Energy minimization and molecular dynamics calculations have been applied to some models, while in a few cases, energy functions were not used owing to concerns about whether those actions improve or degrade models (75) or whether the energy functions are appropriate for proteins in lipid environments (10).

Several of the papers describe comparisons of models with the X-ray crystallographic model. One measure of the fit of two models is the rms distance between equivalent atoms. Some of the models have large rms distances above 3 or 4 Å (31, 76, 99), which are substantially larger than rms distances calculated for structures of homologous soluble proteins obtained from X-ray diffraction studies.

The primary goal of the studies reviewed in the remainder of this article has been to understand a particular GPCR in terms of the rhodopsin structure. Another thing to learn from these studies is whether the structural model of rhodopsin is representative of other GPCRs. All molecular models, regardless of source, need to be consistent with information obtained from complementary approaches (76). Extrapolation of the structure to explain the properties of other GPCRs provides one means of validating the crystallographic model.

Characterization of Ligand Binding Sites

Knowing where ligands bind to a receptor is useful for designing new agonists or antagonists. Much is known about the binding sites in most GPCRs, but the more detailed crystal structure of rhodopsin has provided new views of the site for investigating ligand binding. As expected, the residues implicated in ligand binding are located near the extracellular or intradiscal surface of the receptor. Figure 2 shows the binding pocket residues identified for the angiotensin I receptor located on the rhodopsin crystal structure, as well as residues involved in the protein conformational change and G protein interactions.

One experimental approach for determining which residues contribute to a binding site is the substituted Cys-accessibility method (SCAM). Using mutagenesis techniques, Cys can be substituted at positions in the polypeptide chain, and their accessibility can then be determined by reaction with sulfhydryl reagents in the absence and presence of receptor ligands. In the case of human A₁ adenosine receptor (22), residues T7.35[270], A7.38[273], I7.39[274], T7.42[277], H7.43[278], N7.49[284], and Y7.53[288] were identified as ligand binding residues. The authors then noted the positions of these residues in the rhodopsin structure and concluded that they make up part of a contiguous binding pocket. SCAM was also used to investigate residues of helix VI of the μ , δ , and κ opioid receptors (124). Similarities in the accessibility patterns of these receptors and that of dopamine D2 receptor for helix VI (53) indicate that the secondary and tertiary structures of these receptors are quite similar.

Mapping studies of receptors with multiple binding sites have also been carried out. Allosteric effectors of M₁ muscarinic receptor are known, and two residues have been identified that affect binding of the effector gallamine (14). W3.28[101] and W7.35[400] (muscarinic receptor residue numbers) affect gallamine binding. W7.35[400] is located on the edge of a cleft in the extracellular part of a model obtained by threading the muscarinic receptor sequence onto the rhodopsin structure. W3.28[101] is buried inside the protein, on the other side of the β -strands. It likely plays some role in stabilizing the structure rather than in interacting directly with the allosteric effector.

Models based on the rhodopsin crystal structure have also been reported for GPCRs that respond to small molecule ligands. In the case of α_1 -adrenergic receptor (122), an antagonist binding site has been shown to involve residues F7.35[308] and F7.39[312].

Also, three papers have described differences in the binding of agonists to human and rodent histamine receptors. In one comparing the binding characteristics of human and guinea pig histamine H₂-receptor (55), a homology model based on the rhodopsin structure helped predict that a nonconserved Asp in helix VII was responsible for the binding differences. Mutagenesis of Asp to Ala confirmed this idea. In another study comparing human and rat histamine H₃-receptors (107), an Asp on helix III could account for the difference in the binding profiles for the two receptors. A homology model with docked ligands, including histamine, helped in understanding the differences in binding. Finally, the contributions of residues in helix V to the binding of agonists to human H₃-receptor were investigated (115). In the homology model in this study, helix V had to be manually rotated and positioned to allow interactions between histamine and E5.46[206] that were known from other experiments.

Combinations of mutagenesis techniques, homology modeling, and other biochemical approaches have mapped out the binding sites and characterized several peptide binding GPCRs. Studies making use of the rhodopsin crystal structure have been reported for rat angiotensin II receptor of type 1 (in complex with angiotensin II) (77), bradykinin receptors (68, 88), opioid receptors (19), the cholecystokinin

receptors (24, 29, 37), complement factor 5a receptor (33), human formyl peptide receptor (73), mouse gastrin-releasing peptide receptor (113), parathyroid hormone receptor (74), and human gonadotropin-releasing hormone receptor (50).

In all these cases, the biochemical data were reconciled with the homology models. Access to the ligand binding site and the structure of the extracellular loops are major issues for models of the peptide binding GPCRs because the peptides will likely come from the aqueous phase and the binding site might be large. The authors of these papers dealt with these in different ways. In some of the cases, only the transmembrane helices were included in the homology model. In others, special attention was given to one or more of the loops. Some were modeled by looking for equivalent structures in the Protein Data Bank for the loop peptides, some were modeled using *ab initio* approaches, and some were modeled using NMR-derived structures for peptide segments of the receptors.

The rhodopsin model was used in two studies of melanocortin-4 receptor. Hydrophathy plots based on the sequences of human melanocortin receptors were used to map mutations onto the rhodopsin model to ensure that the sites were in the transmembrane region (125). Residues D3.25[122] and D3.29[126] in helix III and F6.51[261] and H6.54[264] in helix VI decreased the binding of the melanocyte-stimulating hormone. In a study of the mouse receptor (45), several residues involved in agonist and antagonist binding were identified using a model built before the crystallographic structure became available as well as one built using the crystal structure. E2.59[92], D3.25[114], and D3.29[118] are involved in melanocortin-based peptide binding. E2.59[92] and D3.29[118] interact with antagonists. F4.60[176], Y4.63[179], F6.52[254], and F6.57[259] interact with Phe on a melanocortin-based ligand.

Properties and Design of Ligands

Several research groups have used homology models based on the rhodopsin structure for structure/activity studies of GPCR ligands. The receptors involved in these studies include human A₃ adenosine receptor (10, 11), rat serotonin 5-HT₄ receptor (63, 64), and turkey P2Y₁ receptor (56). In the case of the adenosine receptor, a homology model was used in the design of a new ligand (10) that was experimentally proven to be an antagonist of the receptor. While the focus of these kinds of studies is on the properties of the ligands, they also provide insight into the effects of the ligands on receptor structure and signaling. A model of human CXCR4 chemokine receptor (missing the extracellular loop between helices IV and V) was generated for a study of cyclam and cicyclam antagonists (34). This chemokine receptor is a coreceptor for HIV, and the authors proceeded to test the binding of antiviral compounds to it. Docking of AMD3100, an antiviral bicyclam, into the model placed one cyclam close to D4.60[171] and one close to D6.58[262]. The agonist activity is felt to be due to the ligand preventing the receptor from altering its conformation into an active one. This idea of restraining or constraining the structure of the receptor will return in the next portion of this review.

Another example showing the use of a rhodopsin-based homology model in a molecular design process concerns the development of mutant receptors (neoreceptors) with selective binding properties for synthetic ligands (52). The approach used identifies positions in a receptor binding site where an altered ligand can be accommodated by a mutated receptor. In the case reported, a mutant adenosine A₃ receptor (H7.43[272]E) was developed, and the binding of amine-modified nucleosides was measured. A homology model of the neoreceptor was extensively used in this study, which hoped to bind a 3'-amino-3'-deoxyadenosine via a direct electrostatic interaction between the ligand and the substituted glutamic acid. The derivative bound well, but its binding was not explained by the proposed interaction. Instead, loss of repulsion from His is felt to be a more important contribution to the altered binding.

The DRY Motif

Sequence comparisons and other studies have pointed out the existence of a tripeptide sequence, DRY, in helix III of a large number of GPCRs (the residue numbers for these amino acids are D3.49, R3.50, and Y3.51). If the motif is expanded to be (E/D)R(Y/W), the pattern's conservation is greatly extended. The location of the ERY sequence in rhodopsin is shown in Figure 3. The overall picture common to many GPCRs is that R3.50 is hydrogen bonded to a carboxylate side chain at position 3.49 and to one or two residues in helix VI. In rhodopsin, those are E6.30 and T6.34. Removal of these interactions often results in constitutive activation of the receptor.

Several studies combining mutagenesis and homology modeling have attempted to identify the residue(s) interacting with R3.50 and what their physiological effects might be. Groups have investigated this motif in rat μ opioid receptor (51, 62), β_2 -adrenergic receptor (7), α_{1b} -adrenergic receptor (41), lutropin/choriogonadotropin receptor (4), and 5-hydroxytryptamine 2A serotonin receptor (100). Mutagenesis of D3.49 in the opioid receptor results in activation, and neutralization of the charge on E6.30 in the adrenergic receptors does likewise. The current favorite interpretation of these results is that the electrostatic-hydrogen bonding interactions restrain the position of R3.50. Removal of the restraints results in straightening of helix VI and a separation of helices III and VI to form an activated receptor.

As attractive as this bit of molecular machinery is, there are two or three suggestions from other modeling studies that need to be kept in mind when considering the biological function of the DRY motif. First, the charged groups interacting with R3.50 will not be found in all GPCRs. In the case of lutropin receptor (98), mutagenesis methods have ruled out an interaction between R3.50 and Asp meant to substitute for E6.30 in rhodopsin. Second, the Kaposi's sarcoma-associated herpesvirus GPCR (48) has no negatively charged side chains for R3.50. This receptor has high levels of basal activity, and the authors interpret this to mean the receptor can serve as a model of the activated receptor. Third, in the M-3 muscarinic acetylcholine receptor (120), helices V and VI move closer together upon

activation. This, instead of a separation of those helices, may be the real activation movement, but further structural studies are needed to confirm this.

Modeling of Bent Helices

As expected from the extensive sequence comparisons and alignments of GPCRs, the receptors share several three-dimensional structural elements that correlate with similarities in their functions as signaling proteins. One such structural feature concerns the bent helices that help differentiate GPCRs from the other seven transmembrane helical proteins. As described above, six of the transmembrane helices in rhodopsin are bent, with the bending often associated with Pro or a pair of adjacent Gly residues. These residues are often conserved in the GPCR sequences (9, 12, 54, 123), and it is anticipated that as a consequence, the bent helices will also be conserved. Several modeling and computational exercises support this view.

Modeling studies of β_2 -adrenergic receptor (7) included computer simulations of a bend in helix VI of the receptor. While the presence of Pro causes the helix to bend, the amount of bending in the simulations is less than that found in the rhodopsin crystal structure. The authors point out other interactions in the inactive, nonsignaling form of the protein that can constrain the helix to be more bent.

Further simulations of helices containing Pro residues were carried out employing the chemokine receptor CCR5 (39). In this receptor, helix II contains a TXP sequence, and molecular dynamics calculations indicate that the helix could bend at these residues. In fact, the Thr located two residues before Pro increases the bend of the helix by 10° in simulations, presumably owing to hydrogen bonding possibilities between the side chain hydroxyl and the main chain atoms. The corresponding sequence in bovine rhodopsin is GGF, which is another sequence motif associated with bending of helices. It is interesting that the two receptors have different amino acid sequences in this region, both of which support a helical bend. However, the equivalent sequences, although located in the same positions, bend the helices in different directions. In rhodopsin, the helix is bent toward helix I. In the adrenergic receptor, the helix bends toward helix III. It would be interesting to know if this difference is real and how it fits with the idea of "structural mimicry" proposed by Ballesteros et al. (8) as a mechanism for generating selective receptors with a common tertiary structure and signaling mechanism.

A study of human VPAC₁ receptor (58) pointed out another issue about the Pro-kinked helices that requires attention in modeling GPCRs. This receptor is a member of the secretin class of GPCRs. The conserved Pro residues in this class of receptors are located in helices IV, V, and VI, not helices V, VI, and VII as in rhodopsin. This requires special attention in generating homology models because the bent helices are likely to be functionally important. In the case of VPAC₁ receptor, mutations of Pro residues in helices IV, V, and VI affected the properties of the receptor. Alteration of Pro in helix IV reduced the activity of the receptor, while the mutations in helices V and VI increased the ability of the receptor to

initiate cAMP production. In a related study of human prostacyclin receptor (109), it was discovered that mutation of Pro and Ala residues in helices IV, V, and VI reduced ligand binding while similar mutations for Pro residues in helices I, II, III, IV, VI, and VII affected activation of the receptor. Sorting out why these mutations have their different effects requires more research.

For some modeling exercises, straightening of the helices will require consideration when the amino acid sequence contains no special sequence motifs that support bends. Dopamine D2 receptor has no Pro in helix I, and simulations were undertaken to see how the sequence might affect the dynamics of that structural element (101). The Gly residue equivalent to Pro in helix I in rhodopsin gives the dopamine receptor's helix more flexibility than would other amino acid substitutions, but it is not clear whether Gly would permit as much bending as found in rhodopsin.

Transmembrane Helices III, VI, and VII

As described in the discussion of the DRY motif, interactions between helices III and VI are involved in restraining the structure of GPCRs in the inactive, non-signaling state. The set of interacting residues can be expanded beyond R3.50 and its hydrogen-bonded partners to include additional residues, some of which are found in helix VII. The following examples present experimental evidence for interactions among the three helices and how they are associated with the activity of the receptors. What is uncertain is whether these studies on different GPCRs provide information relevant to all GPCRs or whether variation exists among the receptors in the structural elements and interactions giving rise to signaling. At this time, there is no strong evidence requiring radically different activation schemes for different receptors.

One part of the basic interaction between helices III, VI, and VII is illustrated by a study of human B₂ bradykinin receptor (68). In this receptor, W6.48[256] controls the balance between active and inactive conformations. Through its interactions with inverse agonists, it can control the relative motion of the three helices. It was hypothesized that an interaction between N3.35[113] and W6.48[256] would stabilize an inactive conformation. W6.48[256]F and Q mutants were constitutively active, consistent with the hypothesized interaction in the wild-type protein. A lack of constitutive activity for the W6.48[256]A mutant was taken as an indication that additional residues and interactions might be involved in activation of the receptor.

Additional interactions between helix III and helix VI have been described for human lutropin receptor (103). The natural mutant L3.43[457]R for this receptor is constitutively active. This leucine is highly conserved and is located in helix III. Mutagenesis of this residue shows that activation is associated with a positively charged amino acid at this position. Modeling shows that R, K, or H at 3.43[457] interact electrostatically with D6.44[578] in helix VI. This perturbs the interhelical interactions between III, VI, and VII and gives rise to the activity of the receptor.

Further examples can be found where individual parts of helices III, VI, and VII are implicated in activation. In opioid receptors, the major residues involved in antagonist binding are located in helix VI (124). In thyrotropin receptor (40),

an interaction between D6.44[633] and N7.49[674] serves as an on/off switch for activation. Constitutive activity in muscarinic receptors can be generated by mutations of residues in helices III and VI (105). Finally, for C5a receptor, it has been proposed that binding of agonists via interactions with residues on helices III and VII causes them to change in their relative orientations and allow helix VI to separate from them (33).

Activation and Binding of G Proteins and Other Macromolecules

As was stated in the first part of this article, movement of the transmembrane helices is a major component in the transition from an inactive ground state to an active signaling form of the receptor. However, it is not sufficient for binding of G proteins. The cytoplasmic loops of the receptors are also involved in recognition and binding of the G proteins. Using the bovine rhodopsin structure to model the interaction with the G proteins is difficult. The cytoplasmic loops are flexible and disordered in the crystals, enough so that residues in the loop are not seen in the electron density maps. The resulting protein structure cannot provide much information about the structure of the loops relevant for the function of the receptor. Nevertheless, two studies have been reported [one for the V2 vasopressin receptor (28) and one for the α_{1b} -adrenergic receptor (42)] where modeling of the receptors with the cytoplasmic loops started with the rhodopsin crystal structure. The models found some use in the design of mutants, but the range of structures available for the loops complicated the analysis of the mutants.

While interactions between GPCRs and their G proteins are of prime importance, interactions between neighboring receptors have become the object of much speculation and experiment. One group has used modeling techniques to identify regions on the hydrophobic surfaces of GPCRs that might participate in protein-protein interactions leading to the formation of dimers and other oligomers (23, 38). Two sets of surfaces on the proteins, one containing helices II and III and the other containing V and VI, are suggested as regions that might be involved in the formation of hetero- and homodimers.

Investigators are also using molecular structure information in approaching questions concerning expression and localization of GPCRs. For example, differences in expression of murine and human vasopressin V₂ receptors in COS cells are due to a single amino acid change at position 100 at the interface between helix II and the second extracellular loop (83). Examination of a homology model shows that Lys at this position can make hydrogen bonds with residues in helices I, II, and III, whereas Asp (the change leading to reduced expression) would destabilize the protein structure via electrostatic clashes with other carboxylates.

Assessment of Alternative Models

The 2.8 Å resolution crystal structure of bovine rhodopsin has had a major impact on GPCR research, both in providing structural information for modeling and structure analysis, and in showing that structural studies of proteins in this

family are possible. The large number of papers using the experimental structure for modeling studies shows the level of interest in using structural information for understanding GPCR function. In the absence of experimentally determined structures, modeling provides a means of generating that base of structural information. However, it is often difficult to assess the quality and relevance of molecular models. Concerns about the structures of the loops in rhodopsin have been expressed (21, 69), as have questions about the validity of the rhodopsin structure as a general template for GPCRs (25, 76, 77).

There are five papers available at this time (June 2002) containing comparisons between models based on the rhodopsin crystal structure and models obtained using other approaches. In a modeling study of cholecystokinin receptor, the rhodopsin crystal structure was compared with an earlier receptor model generated by the authors (25). Imposition of the receptor sequence on the rhodopsin template introduced many structural clashes between residues within the transmembrane region, and these made its use for further studies unattractive.

Another instance allowing comparison of alternative models is in a paper describing structure/function studies of melanocortin receptor (45). This particular study was already underway before the rhodopsin crystal structure was reported. The investigators were using a homology model to generate lists of possible ligand-receptor interactions. They built another homology model based on the rhodopsin crystal structure and could see no significant differences in the possible ligand-protein interactions. Each model was adequate for this purpose.

Another example where two GPCR models were directly compared was in an investigation of human B₂ bradykinin receptor (68). Again, two models were built. The "experimental model," was built using a previous model as a template. In addition, two of the helices, VI and VII, were rotated slightly in response to binding information from mutant receptors. The "rhodopsin-like model" was built using the crystal structure as a template. Molecular dynamics simulations of ligand binding resulted in different binding modes for each ligand/model pair, along with differences in the predictions about which residues were in contact with the ligand. Resolution of the differences did not favor either model. However, in another part of the study, the proximity of two residues was predicted on the basis of earlier work. Replacement of the two residues with His, followed by exposure of the protein to zinc ions, showed that the residues were close to one another. The results were more in line with the rhodopsin-like model than with earlier published rhodopsin models.

Finally, two examples are available where differences between two structural models were clarified using mutagenesis techniques. In the case of α_{1b} -adrenergic receptor (41), two models were generated, one being a homology model based on the rhodopsin crystal structure and the other being generated ab initio. The prediction from the first model is that R3.50 (in the DRY motif) would interact with E6.30. The ab initio model predicts that R3.50 interacts with D2.50 and Y7.53. Mutagenesis experiments were consistent with the prediction from the homology model based on the rhodopsin structure.

Studies of a possible sodium binding site on dopamine D₂ receptor (75) also provide strong evidence supporting the validity of the rhodopsin crystal structure

as a template for GPCRs. An older model of the receptor indicated that D2.50[80], S3.39[121], N3.42[124], and S7.46[420] form the base of a pyramidal sodium binding site with N7.49[423] at the apex. A new model based on the rhodopsin structure placed N7.45[419] (backbone oxygen) at the apex, with D2.50[80], S3.39[121], N7.45[419], and S7.46[420] at the vertices. Mutagenesis of S3.39[121] and S7.46[420] alters the receptor's dependence on sodium, but mutations of N3.42[124] do not.

CONCLUSIONS

Rhodopsin remains a molecule of importance and interest to two large research areas. The X-ray crystallographic model has been useful for understanding the biochemical and biophysical properties of rhodopsin in the vision system, but it has also proven important for extrapolations into the structure and function of GPCRs. This is consistent with the idea that the basic structure and properties of rhodopsin are representative of proteins in the entire GPCR family. The secondary and tertiary structures of rhodopsin are consistent with experimental information available for other receptors, and the tertiary structure is robust and not overly sensitive to the packing forces it encounters in two-dimensional or three-dimensional crystals. The homology models based on the crystal structure indicate that the details of the helical bends likely apply to many proteins in the family, as do other structural details.

The major structural feature of rhodopsin that likely cannot be carried over directly into studies of other GPCRs is the ligand binding site. Currently, computational techniques appear to be providing suitable alterations in the binding site, but additional three-dimensional crystal structures of GPCRs are needed to determine how varied the binding sites might be. Progress in obtaining additional crystal structures might also provide the strongest evidence that the rhodopsin structure is typical for this class of proteins. One crystallographic technique capable of differentiating between seemingly similar structures such as those of rhodopsin and bacteriorhodopsin is that of molecular replacement. The method provides an orientation and position of a structural model in a new crystal form. It is a powerful technique for determining the structure of a protein related to one with a known structure. In the case of rhodopsin, bacteriorhodopsin and prior models of rhodopsin were inadequate templates for solving the structure. If those techniques can be used with the current rhodopsin model to solve new crystal structures of GPCRs, it will be strong proof of the generality of the structure.

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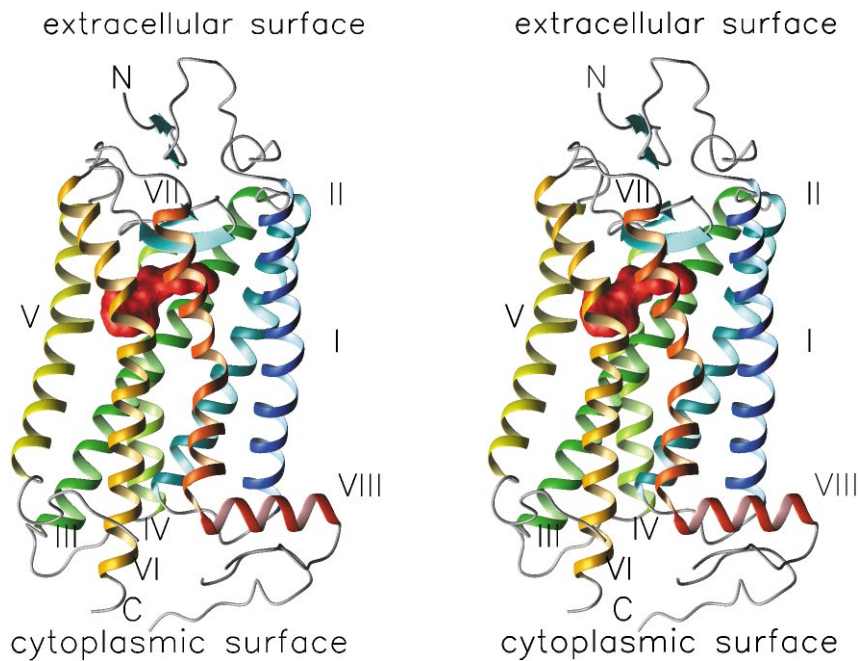


Figure 1 Stereoview showing the secondary and tertiary structure of rhodopsin. The helices are labeled I–VIII. The retinal chromophore is shown in red. The molecule is drawn in the orientation usual for GPCRs with the extracellular surface at the top. Figure drawn using MOLMOL (60).

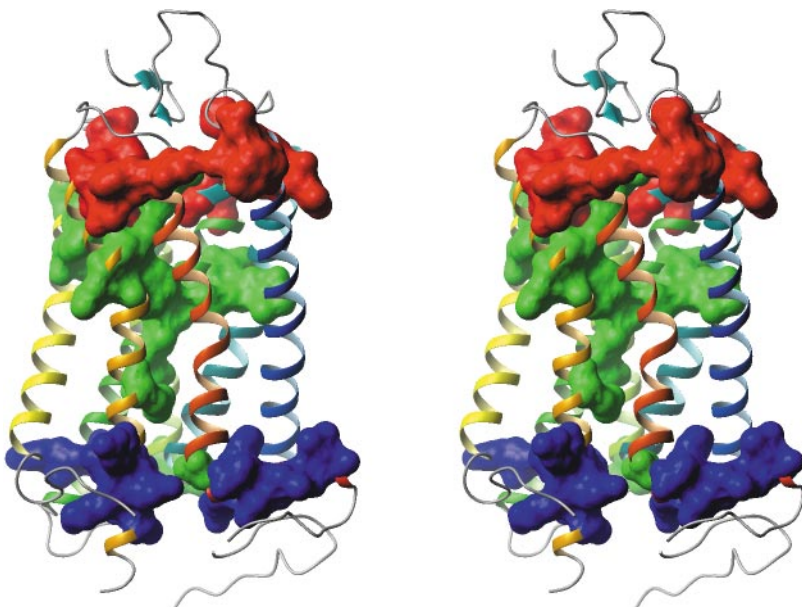


Figure 2 Stereoview showing regions of the at1 angiotensin receptor involved in ligand binding (red), signal propagation (green), and G protein binding (blue). The residues with atoms shown as spheres are from the crystallographic model of rhodopsin, but their positions were chosen on the basis of a tabulation of important residues in the at1 angiotensin receptor (77). Figure drawn using MOLMOL (60).

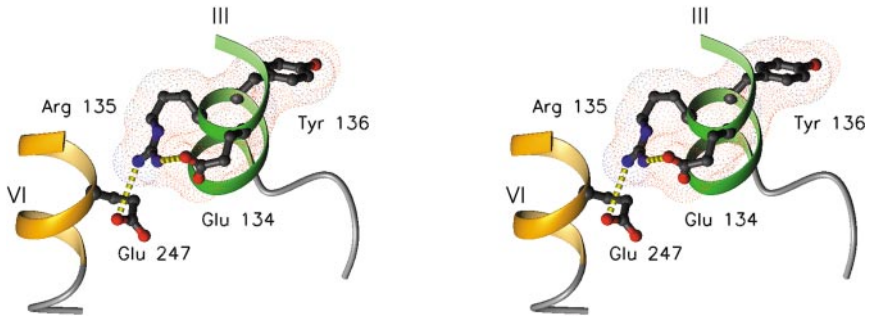


Figure 3 Stereoview of the ERY motif in rhodopsin. E3.49[134], R3.50[135], and Y3.51[136] are shown in ball-and-stick mode enclosed in a van der Waals envelope. E6.30[247] is also shown with its hydrogen bond to R3.50[135]. Figure drawn using MOLMOL (60).