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Review

# G-protein coupled receptor structure

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**Abstract**

Because of their central role in regulation of cellular function, structure/function relationships for G-protein coupled receptors (GPCR) are of vital importance, yet only recently have sufficient data been obtained to begin mapping those relationships. GPCRs regulate a wide range of cellular processes, including the senses of taste, smell, and vision, and control a myriad of intracellular signaling systems in response to external stimuli. Many diseases are linked to GPCRs. A critical need exists for structural information to inform studies on mechanism of receptor action and regulation. X-ray crystal structures of only one GPCR, in an inactive state, have been obtained to date. However considerable structural information for a variety of GPCRs has been obtained using non-crystallographic approaches. This review begins with a review of the very earliest GPCR structural information, mostly derived from rhodopsin. Because of the difficulty in crystallizing GPCRs for X-ray crystallography, the extensive published work utilizing alternative approaches to GPCR structure is reviewed, including determination of three-dimensional structure from sparse constraints. The available X-ray crystallographic analyses on bovine rhodopsin are reviewed as the only available high-resolution structures for any GPCR. Structural information available on ligand binding to several receptors is included. The limited information on excited states of receptors is also reviewed. It is concluded that while considerable basic structural information has been obtained, more data are needed to describe the molecular mechanism of activation of a GPCR.


*Keywords:* GPCR; Structure; Membrane protein

**Contents**



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## 1. Introduction

G-protein coupled receptors (GPCR) function at the heart of inter- and intra-cellular regulation. These transmembrane proteins interact with extracellular signals, usually through the binding of small signaling molecules. This ligand binding induces a change in conformation of the receptor that is transmitted to the cytoplasmic face of the protein, enabling a coupling of the cytoplasmic face with an intracellular heterotrimeric G protein (GTP binding protein). The intracellular G protein, in turn, acts as an intracellular signal by activating or inhibiting intracellular enzymes. This model of cellular communication became so successful through evolution that GPCRs are used to enable the senses of taste, smell, and vision, and to control a myriad of intracellular signaling systems. Nearly 1000 such receptors are thought to be present in the human genome [1]. Diseases such as some forms of blindness, obesity, inflammation, depression, and hypertension, among others, can be linked to malfunctions of GPCRs [2]. Not surprisingly, about half of drug targets in the pharmaceutical industry are GPCRs [3].

The power of structure to inform studies on function has been well demonstrated in many systems. Therefore it is no surprise that enormous effort has been expended to obtain structural information for GPCRs. Yet, to date, X-ray crystallography has provided information on only one GPCR, bovine rhodopsin. The dearth of crystallography on GPCRs is a result of the problems that beset structural studies on all integral membrane proteins: such proteins are insoluble in water and cannot be readily crystallized. Furthermore, nuclear magnetic resonance (NMR) studies on intact membrane proteins are problematic due to the size of the requisite protein-detergent micelle and the influence of the relatively slow rotational correlation time on the relaxation properties.

Therefore in this review, the X-ray crystal structure of rhodopsin will be discussed, but much of the review will address alternate approaches to structure of GPCRs because for all GPCRs other than rhodopsin, no crystal structures are available and the alternate approaches are the only path to structural information.

## 2. Early structural information on rhodopsin

Rhodopsin was the first GPCR to be studied in detail. The ability to isolate rhodopsin in milligram quantities from bovine retinas enabled a wide variety of biochemical and structural experiments well before studies on other GPCRs commenced, in fact before it was known that rhodopsin was a member of the superfamily of GPCRs. While it has been known for a long time that GPCRs are built around a bundle of 7 transmembrane helices, it is valuable to understand from what experiments this knowledge was first derived.

Circular dichroism (CD) experiments in the 1960s and 1970s were widely used to investigate secondary structure in soluble proteins. These optically active transitions were shown to be sensitive to the presence of α-helix, β-sheet, etc., in the protein structure. Consequently, CD was also used to explore membrane protein structure. For example, the secondary structure of human erythrocyte glycophorin was examined with CD, both by CD measurements on the intact protein and by CD measurements on proteolytic fragments of glycophorin. Not only did the data show that the transmembrane segment of this protein was α-helical, but these experiments also revealed a domain structure in this membrane protein: the fragments of the protein retained the secondary structure characteristic of the intact protein [4]. This domain property of membrane proteins has been exploited in structural studies of GPCRs, as will become apparent later.

CD studies on rhodopsin from the University of Virginia in 1978 were the first to provide quantitative evidence for a transmembrane helical bundle as an architectural feature of this GPCR. CD studies on rhodopsin in rod outer segment disks and purified rhodopsin in octyl glucoside micelles indicated a largely α-helical structure. When the α-helical content was analyzed, the α-helical content was consistent with about 7–9 transmembrane helical segments [5]. Combining this with a knowledge that the amino terminal and the carboxyl terminal were on opposite sides of the membrane [6] led to the initial suggestion that rhodopsin was built on a bundle of 7 transmembrane helices.

The next major step in the search for rhodopsin structure was the publication from Moscow and from the University of Southern Illinois in 1982–83 of the primary sequence of rhodopsin [7,8] from chemical sequencing experiments. This represented a much larger challenge in that time than sequencing membrane proteins presents today since in the intervening period the ability to sequence from the DNA became feasible. With the primary sequence in hand, a hydropathy plot could be obtained of the protein. Such a plot

is shown in Fig. 1 for the 348 residues of the bovine form. The hydropathy plot shows six clear hydrophobic transmembrane segments. The hydropathy plot is not as clear for the seventh transmembrane segment because of the presence of the lysine in the middle of that segment.

It was 10 years before the next major breakthrough occurred in the search for the first structure of a GPCR, rhodopsin. Two-dimensional crystals of dark-adapted bovine rhodopsin were obtained at the University of Cambridge in 1993. Cryo-electron microscopy from tilted specimens yielded a low resolution two-dimensional projection map of rhodopsin in phospholipid bilayers [9]. In these maps one could see unequivocally the bundle of seven transmembrane helices that are now believed to characterize the structure of all GPCRs (see for example [10]). An expanded set of data were subsequently utilized to obtain limited projections in a third dimension, revealing for the first time the overall shape of the rhodopsin molecule [11]. Many, but not all, of the transmembrane helices were seen to lie approximately perpendicular to the membrane surface.

In 2000, the first three dimensional crystals of bovine rhodopsin were obtained [12]. These quickly led to a three dimensional high resolution structure for this GPCR, which for the first time provided a sufficiently detailed view that the disposition of the retinal in the structure could be determined [13]. This review will return to further discussion of these and other landmark reports that have provided a detailed view of the structure of bovine rhodopsin, still the only GPCR for which high-resolution structures are available.

## 3. Alternative approaches to GPCR structure—studies of fragments

The path to a three dimensional structure of rhodopsin was long and difficult. To date, no other X-ray crystal structures of GPCRs have been published. The difficulty arises from the inherent problems with crystallization of membrane proteins. Membrane proteins must be isolated in detergents because of the substantial portion of the protein surface that is hydrophobic (the transmembrane domain), and the detergents in a sense constitute an "impurity" which inhibits crystallization generally. Furthermore for many membrane proteins much of the protein surface is covered by the detergent prohibiting the kinds of protein–protein contacts characteristic of three-dimensional crystals. The magnitude of the difficulty posed by these and other factors is manifested by less than 0.5% of the entries in the Protein Data Bank that report structures of membrane proteins.

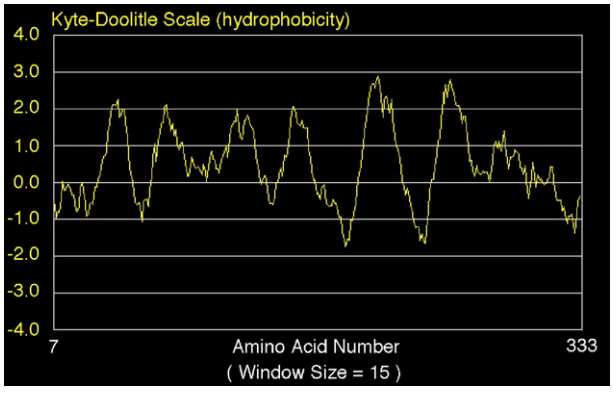


Fig. 1. Hydropathy plot for rhodopsin, using http://www.vivo.colostate.edu/molkit/hydropathy/index.html.

The other major tool for determination of high-resolution structures is NMR. While this approach has been very successful for soluble proteins, only a few structures of membrane proteins have been obtained by NMR. These are the porins from bacteria, which are β-barrels. No structures have yet been obtained for proteins built around bundles of transmembrane helices. The reasons are several-fold. Since membrane proteins must be purified in detergents, it is a complex of detergents and protein that must be studied. The complex is large, effectively many 10s of kDa, and the rotational correlation time of such a complex is long relative to many soluble proteins, leading to unfavorable $T_2$ relaxation and broad overlapping resonances. While new techniques offer a partial solution [14], another problem intervenes. Deuteration is required to help defeat the relaxation problems, and is achieved, in part, by expressing the protein in a system containing $D_2O$. This leads to deuteration of key-NH on the polypeptide backbone, which must be exchanged for hydrogen, $^1H$. The β-barrels of the porins can be sufficiently unfolded to permit exchange while allowing refolding to the native configuration. However, reversible unfolding of the α-helices of transmembrane proteins in detergents sufficient to permit hydrogen exchange as well as refolding to the native structure is problematic.

Therefore alternative approaches to the structures of GPCRs are, and will continue to be, essential. One very useful approach is the study of suitably chosen fragments of membrane proteins. For example, biochemical experiments demonstrated that peptide fragments from the cytoplasmic face of the GPCR, rhodopsin, inhibited the interaction of rhodopsin with its G protein, transducin [15]. This observation was the inspiration for a study of one such peptide fragment by solution NMR. The rationale was that if the peptide fragment of the receptor had such biological activity, and if the peptide fragment also was structured in solution, then the solution structure was likely relevant to the structure of the native protein. In fact, the first such NMR study in 1995 discovered that one of those rhodopsin peptides with biological activity was structured in solution, initially revealed by CD measurements, and subsequently defined in detail by high resolution solution NMR techniques [16].

CD was also the method of choice for an early study of fragments of the avian and mammalian β-adrenergic receptors. The investigators reported that fragments of the third cytoplasmic loop interacted with membranes and adopted increased helicity when bound [17].

These observations was quickly followed by an NMR study of a peptide fragment from the cytoplasmic face of another receptor, the G-protein coupled parathyroid hormone/parathyroid hormone related protein receptor, which also proved to be structured in solution [18]. In the same time period, a peptide corresponding to the seventh transmembrane domain of the

tachykinin NK-1 receptor was synthesized and studied by solution NMR. While no biological activity was ascribed to this segment to link it with the intact receptor, the structure obtained was helical [19], which proved to be completely consistent with the subsequent structural work. These studies opened a new field of research in which fragments of GPCRs were synthesized or expressed and their structures determined by solution NMR techniques. This approach has offered the widest range of structural information on GPCRs to date and will be reviewed in the following, organized by receptor.

### 3.1. Angiotensin II AT1A receptor

The rat angiotensin II AT1A receptor is a GPCR. The third cytoplasmic loop, the first extracellular loop and a portion of the carboxyl terminus of this receptor have been studied as peptides in solution with NMR. A peptide fragment, corresponding to residues 300 to 320 of the whole receptor, was studied by solution NMR. The data showed that part of this fragment formed an amphipathic helix [20]. The X-ray crystal structure of rhodopsin suggested that this amphipathic helix likely corresponded to what is now called helix 8 which may be involved in binding to the G protein [21]. Two peptides were synthesized that spanned the third cytoplasmic loop of this receptor [22]. While only modest structure was observed in these fragments, nevertheless some helix was observed. While in one of the peptides the helix likely reflects an extension of the transmembrane helices of the receptor, helix in the other peptide most likely represents a helical region involved in binding to the G protein [23]. The fragment peptide corresponding to the first extracellular loop forms a type 2 β turn [24] which is stabilized by the presence of membranes [25]. As will be seen in this section, many of the small turns of these GPCRs (connecting two transmembrane helices) are stable as turns separate from the remainder of the protein. This observation is consistent with calorimetric experiments that demonstrate a modest contribution to overall protein stability from the loops of integral membrane proteins consisting of transmembrane helical bundles [26].

### 3.2. Parathyroid hormone receptor

Fragments of the parathyroid hormone receptor, a GPCR, have been studied by solution NMR techniques in detergent micelles. A peptide corresponding to the third cytoplasmic loop of this receptor was synthesized and the NMR solution structure determined in sodium dodecylsulfate (SDS) micelles and in dodecylphosphocholine (DPC) micelles. As a part of the pattern referred to above with fragments corresponding to loops on the face of GPCRs, the peptide corresponding to the loop region formed a loop structure in the presence of detergent micelles and in the absence of the remainder of the protein [18,27]. A peptide containing the amino acid sequence of the first extracellular loop was also synthesized. A short helix was seen in the NMR structure on each end of the peptide corresponding to portions of the TM on either side of the loop. The interior of the loop also contained an additional helix [28].

### 3.3. The human cannabinoid receptor

The human cannabinoid 1 receptor is the receptor that binds $\Delta^9$ tetrahydrocannabinol and is coupled to $G_{i/o}$. A 44-residue peptide has been expressed containing the amino acid sequence of the third cytoplasmic loop of this GPCR. The peptide is biologically active, thereby exhibiting a similarity to the behavior of peptide fragments from the cytoplasmic face of rhodopsin. The solution structure of this peptide exhibits helix at both ends of the peptide, corresponding to portions of the two connected transmembrane helices. The peptide forms a turn in detergent micelles [29] mimicking the turn found in the intact protein. A peptide fragment corresponding to helix 8 of the receptor was studied in the presence of DPC micelles and also in SDS micelles and found to be predominantly helical [30]. The putative helix 8 of the cannabinoid 2 receptor has also been synthesized and the structure determined in DPC micelles and in DMSO. In both environments an α-helix was observed [31].

### 3.4. The human cholecystokinin-2 receptor

The human cholecystokinin-2 receptor from the gastrointestinal tract and central nervous system binds cholecystokinin in the extracellular face of the receptor. The third extracellular loop of the cholecystokinin-2 receptor (residues 352–379), a GPCR, was synthesized and its structure determined in detergent dodecylphosphocholine (DPC) micelles by solution NMR. The ends of the two helices, 6 and 7, to which the loop is attached are seen as is the turn connecting the helices [32]. Interactions between this third extracellular loop and ligands have been probed by NMR and other techniques exploiting the remarkable observation that the peptide fragment corresponding to the third extracellular loop forms a stable complex with the hormone [32–36].

### 3.5. Bradykinin B2 receptor

A 34-residue peptide fragment corresponding to the second intracellular loop of the bradykinin B2 receptor was synthesized and the structure determined by solution NMR. A helix–turn–helix motif was observed, a repeating theme for peptide fragments corresponding to loops of GPCRs. The N-terminal helix and the C-terminal helix of this peptide fragment likely corresponded to the ends of the transmembrane helices to which this loop is attached. The structure of a portion of the C-terminus of this receptor was examined using a fragment that was expressed and stable-isotope labeled and contained residues 309 to 366 of the receptor. Evidence for helical structure corresponding to helix 8 of GPCRs was observed in the structure of this fragment of the bradykinin receptor [37,38].

### 3.6. Neurokinin-1 receptor

The neurokinin-1 receptor is expressed in neural tissue and binds neuropeptide substance P (SP). A peptide fragment of the amino terminus of this protein was synthesized and the structure determined in the presence of DPC micelles. Some helix was

observed, likely a portion of TM1. As a companion study a 27 residue peptide corresponding to the third extracellular loop of this GPCR was made and its structure determined by NMR, showing a largely helical structure, consistent with a helix–turn–helix motif [39]. A fragment corresponding to the second extracellular loop also showed some helical content [40].

### 3.7. β-adrenergic receptor

The β-adrenergic receptor is a GPCR responsive to external hormone levels. Peptide fragments of this protein, corresponding to the third intracellular loop of the turkey receptor (residues 284 to 295), were synthesized and studied in lysophospholipid micelles by solution NMR. The C-terminal region of this loop region showed helical structure, likely corresponding to the beginning of TM 6 [41]. What was originally called the fourth cytoplasmic loop (between TM7 and the palmitoylation site(s) of the GPCR) was also examined as a protein fragment, corresponding to residues 345 to 359 of the turkey receptor. A predominantly α-helical conformation was observed [42]. The putative helix 8 region of the human β-adrenergic receptor was examined with a peptide in detergent and in DMSO. In agreement with the previous study on the turkey receptor, it was also found to be helical. However in water the peptide was disordered [43].

### 3.8. Saccharomyces cerevisiae α-factor receptor

The *Saccharomyces cerevisiae* α-factor receptor is a G-protein coupled receptor of yeast involved in mating. An extensive set of studies of the structure of this protein through the use of peptide fragments has been reported. Peptides corresponding to all the TM of this 7TM transmembrane protein were synthesized and some of the loops. Structures were determined in organic solvent by NMR. All TM showed helical structures and one of the loop peptides was also structured [44–48]. The fragment approach has been stretched through the study of a large fragment of this receptor containing the third extracellular loop, the seventh transmembrane helix, and a portion of the C-terminus [49]. This structure showed helical components in all three portions of the receptor.

### 3.9. α2A adrenergic receptor

The second cytoplasmic loop of this receptor was studied using a peptide fragment in DPC micelles. This peptide proved to be predominantly helical [50].

### 3.10. Human adenosine A2a receptor

Structures of peptide fragments corresponding to all 7 transmembrane domains were determined and found to be independently stable as helices [51].

### 3.11. $V_{1A}$ vasopressin receptor

The $V_{1A}$ vasopressin receptor couples to the αq/α11 G proteins and activate phospholipase Cβ. The second cytoplasmic loop, $iC_2$, plays a critical role in this process. Accordingly, a peptide fragment containing the sequence of $iC_2$ was synthesized, both as a linear peptide and with an extension to connect the amino and carboxyl terminal ends, and both forms inhibited hormone binding to the receptor. Both fragments adopted a helix–turn–helix motif, consistent with a turn in the protein connecting two transmembrane helices [52]. The structures of both peptide fragments were very similar to the structure of the corresponding loop on rhodopsin [53].

### 3.12. Thromboxane $A_2$ receptor

The thromboxane $A_2$ receptor binds the prostanoid, thromboxane $A_2$, and regulates smooth muscle function and hemostasis. To obtain structural information about the extracellular face of this GPCR that binds the prostanoid, peptide fragments of the first and second extracellular loops were synthesized. In each case, loop structures were obtained [54,55], consistent with the connection of this fragment to two transmembrane helices.

### 3.13. Rhodopsin

Rhodopsin is the prototypical GPCR, the first to be studied in detail, and the first to have a crystal structure reported. Rhodopsin is the photopigment of retinal rod cells and responds to light, enabling black/white vision at low light levels. Peptide fragments have been used to scan the entire sequence of the protein to define local secondary structure throughout this receptor. Fragments corresponding to loops (connecting two transmembrane helices) of rhodopsin formed loops in solution [53,56]. Fragments corresponding to the seven transmembrane segments of rhodopsin formed helices [57–59]. Structure was also observed in fragments containing the sequence of the carboxyl terminal of the protein [16,60]. Of particular interest is a recent report of the behavior of a fragment corresponding to helix 8 (see below) of rhodopsin. This region was shown to be non-helical in water while it was helical in the crystal structure. Now it has been shown that this region of rhodopsin acts as a conformational switch: a fragment corresponding to helix 8 was helical only in the presence of membranes [61].

One question that arises is the extent to which structures of receptor fragments reflect the secondary structure of the native protein. This question has been directly addressed using bacteriorhodopsin. A series of peptide fragments of the protein were designed, each overlapping their neighbor in the sequence by about 10 residues. Each fragment represented either a turn or a transmembrane helix of bacteriorhodopsin. High-resolution NMR structures were obtained from each fragment and the structures were overlaid on the corresponding part of the X-ray crystal structure. Good agreement was observed between helices in the protein and the structures of the fragments, and turns in the protein and the structures of the fragments [62,63]. Extensive studies have led to the conclusion that when a fragment of a membrane protein exhibits secondary structure, that structure is similar to the structure in the intact protein. In the case where the fragment is disordered, no conclusion can be drawn.

Another question arises from the study of loops of GPCRs as fragments: will peptide fragments containing the sequences of loops form loop structures without constraints on the position of the amino and carboxyl termini? In some cases, loops naturally formed with free amino and carboxyl termini (see for example, [39,52]. In these cases, the residues in the turn likely directed the form of the structure, perhaps enhanced by helix–helix interactions when the structure was defined by a helix–turn–helix motif. In some examples, β-turns formed which were stabilized by internal hydrogen bonding [53]. In other cases, covalently linking the two ends of the turn enhanced the stability of the turn (see for example, [27,54]). Finally, structural studies of loops in the presence of detergent micelles as a membrane mimetic also stabilized the structures (see for example, [39,50]). Perhaps less surprisingly, the peptide fragments containing sequences of transmembrane helices of GPCRs formed stable helices separate of the remainder of the protein (see for example, [51,59]).

In companion experiments, it is possible to study fragments of G protein binding to the relevant GPCR. One such example is the binding of a peptide fragment of transducin (visual G protein) to rhodopsin. NMR experiments defined the conformation and ultimately the orientation of this fragment when bound to the active form of the receptor [64–66]. This places important constraints on the structure of the complex of the G protein and the receptor.

Although not GPCRs, it is worth noting that this approach to membrane protein structure has been used with success by investigators interested in other membrane proteins as well. Structures of peptide fragments of the following proteins have been determined and found to report on the secondary structure of the parent protein: EmrE, a multidrug resistance protein [67], the potassium ion channel [68], the human red cell anion transporter, band 3 [69,70], the voltage-gated potassium channel, Isk [71], bacteriorhodopsin [72–75], and human erythrocyte glycophorin [76].

## 4. Interactions between transmembrane helices of GPCRs

The work described above on peptide fragments of GPCRs derived from transmembrane segments of the protein revealed that the transmembrane helices of GPCRs are intrinsically stable in their helical secondary structure, a stability apparently arising from their amino acid sequences. These results raise the question of the role of helix–helix interactions in GPCRs. Early experiments with a simple membrane protein, human erythrocyte glycophorin, identified some principles governing helix–helix interactions in membrane proteins [76]. The transmembrane helix of glycophorin forms stable dimers in the membrane. Glycines at the point of closest contact can be important to that stability.

Helix–helix interactions have been discovered that contribute to the stability of GPCRs. Co-expression of bundles of transmembrane helices of rhodopsin (3 TM and 4 TM, or 5 TM and 2 TM) can result in correct reassembly of the fragments into a functional pigment [77]. This remarkable result identifies a strong and specific interaction within the bundle of transmembrane helices. Calorimetry has shown more quantitatively the contribution of these interactions to rhodopsin stability [78]. Such helix–helix interactions were studied in detail for the adenosine $A_{2A}$ receptor. These studies reported that helix stability was enhanced in some cases by the presence of neighboring helices during protein folding [79]. These authors also found evidence for self-association of transmembrane helix 5, which could be part of the foundation for dimerization of receptors [80].

These studies suggest that stability and folding of GPCRs are influenced by several factors. The helical transmembrane segments of GPCRs have considerable local stability and will spontaneously fold, likely early in the folding process [81]. Helix–helix interactions are specific and strong as reflected in the ability of separately expressed bundles of GPCR transmembrane helices to associate correctly. Loops connecting the transmembrane helices in many cases exhibit intrinsic stability and that also contributes to overall GPCR stability. Therefore the stability of GPCRs is built from short-range intrinsic stability of secondary structure (helices and turns) and from helix–helix interactions within the membrane bilayer.

## 5. Modeling of GPCRs

Many groups have, over the years, reported various approaches to modeling GPCR structure driven by the lack of experimental three-dimensional structural information. Most of the early work was done modeling rhodopsin. After the rhodopsin crystal structure was reported, modeling shifted to other GPCRs, for which there are no X-ray crystal structures at the time of this writing. A review of structure modeling for GPCRs is beyond the scope of this review. However, some of the milestones that informed this modeling and some of the pitfalls are important to note. The latter add emphasis to the need for experimental data on GPCR three-dimensional structure.

The first transmembrane protein for which experimental three dimensional structural information became available was bacteriorhodopsin [82–84]. Bacteriorhodopsin was found to consist of a bundle of 7 transmembrane helices, most approximately perpendicular to the membrane surface. The bundle of 7 TM attracted considerable interest for those interested in GPCRs when it became clear that GPCRs also were built around a bundle of 7 transmembrane helices. Many investigators then used the helices of bacteriorhodopsin as a template upon which to build models of rhodopsin. However, it later became known that the arrangement of the transmembrane helices in rhodopsin was not the same as the arrangement in bacteriorhodopsin [85]. Therefore models of rhodopsin built on the bacteriorhodopsin model could not be correct.

Subsequently, an advance in modeling GPCRs was provided by a study from Baldwin [86], in which the available knowledge from sequence and homology was utilized in conjunction with some important new principles for membrane protein structure to produce a model for rhodopsin. Those principles included: conserved residues likely faced other protein surfaces rather than the lipid bilayer and residues with some polar character

likely faced other protein surfaces rather than the lipid bilayer. Combination of such principles with the hydrophobicity plot of rhodopsin allowed a packing of the transmembrane helices in a bundle. This model ultimately proved to be remarkably accurate in its predictions. Using Baldwin's model and some available experimental constraints, a molecular model of the transmembrane helical bundle was developed [87] that ultimately was in reasonable agreement with the X-ray crystal structure. More recent modeling on rhodopsin has explored the use of a wider range of experimental distance constraints to drive the modeling process. This has led to models of metarhodopsin II [88], for example, with considerable detail and with a close correspondence to the experimental approach described above using sparse constraints for structure determination.

More recently, many new modeling approaches have been developed to expand modeling from rhodopsin to other GPCRs (see for example, [89–91]). These have relied on the assumption that all GPCRs have similar structures in the transmembrane domain [92]. Comparisons among rhodopsins provide some justification for this assumption, but also some caution [85]. While the arrangement of the transmembrane helices of bovine, frog, and squid rhodopsins are similar, they are not identical. Therefore some care would be appropriate in extrapolating the arrangement of transmembrane helices in rhodopsin to other members of the rhodopsin family of GPCRs. Even greater care would be warranted if one extrapolated the structure of rhodopsin to the modeling of GPCRs outside the rhodopsin family.

## 6. Structure studies of retinal binding to rhodopsin

The most detailed structural studies of ligand binding to GPCRs have been of retinal bound as a Schiff base in rhodopsin, but it should be noted that there is a large literature of retinal bound to bacteriorhodopsin, an early example of which was performed utilizing neutron diffraction [93]. However this review is focused on GPCRs and therefore will be unable to examine that literature more carefully. And while studies on retinal bound to rhodopsin could be the subject of a review in itself, nevertheless a synopsis of some of that work will be reviewed below as it relates to structure of GPCRs. NMR, particularly solid state NMR, has proven particularly valuable in the study of retinal bound to rhodopsin. As is now well known, retinal is bound as a Schiff base to K248 on TM7 of rhodopsin. The state of protonation of the Schiff base has been the subject of NMR studies with early solid state $^{13}C$ NMR studies of $^{13}C$ labeled retinal and the data were consistent with a protonated Schiff base [94], in agreement with previous spectroscopic studies [95].

How retinal fits into its binding site in rhodopsin was rather completely worked out without the X-ray crystal structure. Retinal as a ligand can be removed from rhodopsin and replaced, and rhodopsin regenerated. Regeneration allows the incorporation of retinal into rhodopsin that has been artificially labeled with stable, NMR-sensitive isotopes. $^{13}C$- and $^{2}H$-labeled retinals were both used in solid-state NMR experiments that allowed the visualization of resonances from the retinal in the binding site in the protein that in normal high resolution experiments would be too broad to be observed. These experiments are largely done at low temperature, both to trap the appropriate state of the receptor and to defeat motional damping that would otherwise obscure the resonances at physiological temperature.

Several groups have contributed studies using this experimental approach. While they do not all agree in every aspect, nevertheless they collectively provide considerable detail on the arrangement of the ligand, retinal in this case, in the protein. The published studies primarily explore rhodopsin.

The orientation of retinal in the protein, relative to the normal of the bilayer, was determined by $^{2}H$ NMR of specifically deuterated retinal with which the rhodopsin had been regenerated [96]; later studies extended this understanding of retinal positioning in the protein [97]. Computational studies synthesized the NMR data into a consensus orientation [98]. This orientation of the retinal later proved to be largely confirmed by the X-ray crystal structure. Such agreement provides a basis for using the same approach in the study of ligands bound to other receptors. The orientation of the retinal is presented in Fig. 2 from the crystal structure of rhodopsin.

Other studies with solid state $^{13}C$ NMR of $^{13}C$ labeled retinals in rhodopsin have provided insight into the photochemistry of this ligand. 11-*cis* retinal photoisomerizes to all-*trans* retinal upon absorption of a photon of light. In ways not yet fully understood (see Activation of rhodopsin below), that isomerization is transmitted to the protein such that the conformation of the cytoplasmic face of the protein changes significantly and binds the G protein. (For other GPCRs, the binding of the ligand induces the analogous conformational change.) Therefore both the conformational response of the retinal and the interactions between the retinal and the protein are of great importance. The H–$C_{10}$–$C_{11}$–H torsional angle of the retinylidene chromophore in rhodopsin was determined to

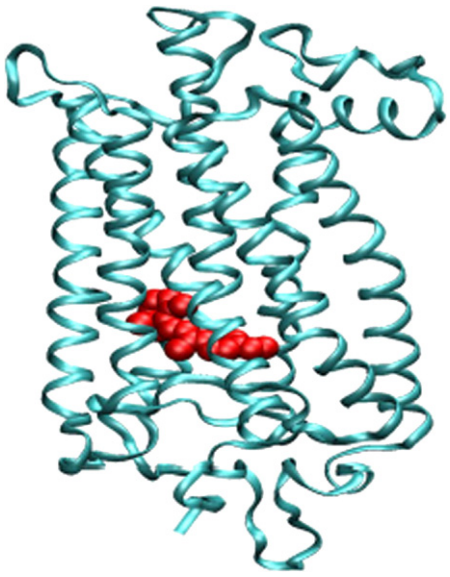

Fig. 2. Orientation of retinal in ground state rhodopsin, from 1U19 [176]. Retinal shown in red. Figure prepared from VMD [177].

be 160°+/−10; this implies a twisted ligand in the ground state [99]. $^{13}C$ chemical shifts strongly reflect electron distribution in the molecule and are particularly sensitive in a highly unsaturated ligand like retinal. Those $^{13}C$ chemical shifts reveal how amino acid residues on the protein that interact with the retinal perturb the retinal structure. An example can be seen in one study of a perturbation in the vicinity of position 13 [100]. The retinal electronic study is strongly influenced by the proximity of counterions to the Schiff base. The latest theories suggest a counter-ion switch: E113 is the ground state counterion [101], but during the photocycle there is a switch to E181 [102,103]. Changes in the position of these charges relative to the conjugated double bonds of the retinal changes the electronic structure of the molecular orbitals and thus the character of the absorption of light.

Other studies provided insight into other contacts between the retinal and the protein. For example, using a uniformly $^{13}C$ labeled retinal and solid state NMR, interactions between the retinal and F208, F212, and W265 were discovered [104]. The W265 interaction with $C_{20}$ of the retinal was suggested to stabilize the ground (inactive) state of rhodopsin [105]. Another solid state NMR study found interactions between Y268 and Y191 and retinal in the binding site [106]. As will be seen later some of these interactions changed after photoexcitation.

## 7. Structural studies of ligand binding to GPCRs

A number of other studies have been reported on the nature of other ligand binding sites on other GPCRs. The richness of the structural information obtained from studies of peptide fragments of membrane proteins stimulated some investigators to push further by posing questions of ligand binding to the receptors. This requires reconstruction of a face of a receptor from the constituent loops. One early example was a reconstruction of the cytoplasmic face of rhodopsin [107]. However, for ligand binding, the extracellular face must be reconstructed, in part or in whole, in the presence of the relevant ligand. Several successful examples have been reported of such ligand–receptor binding, including binding of ligand to the thromoxane $A_2$ receptor [108], substance P binding to the neurokinin-1 receptor [39], and cholecystokinin-8 binding to the cholecystokinin A receptor [35,36,109]. These are very interesting studies in that they imply that structural features of the binding sites are preserved in peptide fragments of the protein; in particular peptide fragments of the extracellular face of the receptor. In each case, the affinity of the ligand for the peptide fragments is sufficient to enable inter-molecular NOEs to be observed that define the contact points between the fragments and the ligand.

## 8. Diffraction studies of 2D crystals of ground state rhodopsin

The goal of the structural studies is ultimately a three dimensional picture of the complete structure of the GPCR. This requires studies of the intact protein. Rhodopsin was the first GPCR to be studied in this fashion because it is the only GPCR that can be readily obtained from tissues in high abundance [110]. Although many attempts have been made to obtain crystals from a wide range of GPCRs, the only successful structural studies to date are of rhodopsin. The following will review the progress, starting with two-dimensional crystals of rhodopsin.

Following the success of Henderson's structure analysis of bacteriorhodopsin in two dimensional crystals [111], early efforts were devoted to developing two dimensional crystals for bovine rhodopsin. The first projection density map for rhodopsin from such two dimensional crystals appeared in 1993 using cryo-electron microscopy [9]. In this map at 9 Å resolution, four regions of well-defined density suggested four transmembrane helices roughly perpendicular to the membrane surface. In addition an arc of unresolved density suggested the location of the remaining three transmembrane helices that were not perpendicular to the membrane surface. As mentioned above, this was critical early evidence for the now well-known 7 transmembrane helical bundle that is the structure around which all GPCRs are likely built.

Schertler took the lead on development of this approach for rhodopsin and progressed by 1995 to a low resolution three dimensional map for bovine rhodopsin [112]. The resolution in the plane parallel to the presumed membrane surface was 9.5 Å and in the plane perpendicular the resolution was about 47 Å. Even with this low resolution, it was now possible to trace several of the transmembrane helices in the transmembrane bundle. At this point it also became clear that the arrangement of the helices was different than reported earlier for bacteriorhodopsin. Therefore while the motif of 7 transmembrane helices was similar between bacteriorhodopsin and rhodopsin, the structure of the two proteins was significantly different. The resolution of this approach was improved with new crystals in 1998 [113].

In concert with these studies on bovine rhodopsin, a report was published on frog rhodopsin that showed the preservation of the configuration of the 7 transmembrane helices from one species to another [114]. An improved picture of the disposition of the helices perpendicular to the membrane surface was obtained and the tilt of helix 3 within the transmembrane bundle became apparent. Another species was added in 1996, squid rhodopsin [85]. At this point, it was possible to compare rhodopsin from three different species and conclude that the arrangement of transmembrane helices was similar in all three species.

Analysis of two-dimensional crystals held a singular advantage over the studies of three-dimensional crystals to follow; the two-dimensional crystals were derived from membrane structures containing lipid. Therefore the resulting structure reflects the influence of the lipid bilayer on structure and likely offers a more accurate view of receptor structure. The singular disadvantage of the two dimensional crystal studies was the relatively low resolution that characterized the 2 and 3 dimensional projection maps.

The resolution from the two-dimensional crystals was improved to 5.5 Å in the plane parallel to the surface of the membrane and 13 Å perpendicular to that plane [115]. This structure identifies unambiguously the orientation of ground state rhodopsin relative to the membrane, information that is not available from the three dimensional crystal structures.

## 9. Diffraction studies of 3D crystals of ground state rhodopsin

The studies just reviewed utilizing electron diffraction of two dimensional crystals of ground state bovine rhodopsin provided critical new information on the arrangement of the transmembrane helices of rhodopsin, but no information on the disposition of the ligand, retinal, or the individual amino acids in the protein. That information awaited the development of three-dimensional crystals of rhodopsin.

In 2000, Okada et al. reported the first diffraction from three dimensional crystals of rhodopsin suitable for high resolution structure determination (see Fig. 3) [116]. This was quickly followed by a full analysis providing the first high resolution three dimensional structure of a GPCR to 2.8 Å [13]. It is fair to say that this report of the structure of bovine rhodopsin completely changed the GPCR field. In this structure, the seven transmembrane helices could be traced fully. Many, though not all, of the loops were identified. The C-terminal region was largely undefined, except for a somewhat surprising helix, called helix 8, appearing at right angles to transmembrane helix 7 (to be discussed further below). At the end of helix 8, the palmitoylation sites are found. The amino terminus, which contains the covalently attached carbohydrate, forms some β-structure that appears to cover the retinal binding site. The retinal can also be seen, bound as a Schiff base to K248 on transmembrane helix 7 and extending roughly parallel to the putative membrane surface through the bundle of 7 transmembrane helices. The interactions of amino acid side chains with the retinal can be seen, including W265 interacting near the β-ionone ring and the counter ion to the Schiff base, E113, all of which were implicated by other experiments as described above. What came to be termed the ionic lock, between R135 (of the highly conserved D(E)RY sequence at the end of helix 3) and E247 on helix 6 [117,118], can be seen in the crystal structure and stabilizes the ground state of rhodopsin. This wealth of structural information from the first crystal structure of bovine rhodopsin stimulated a wide range of studies.

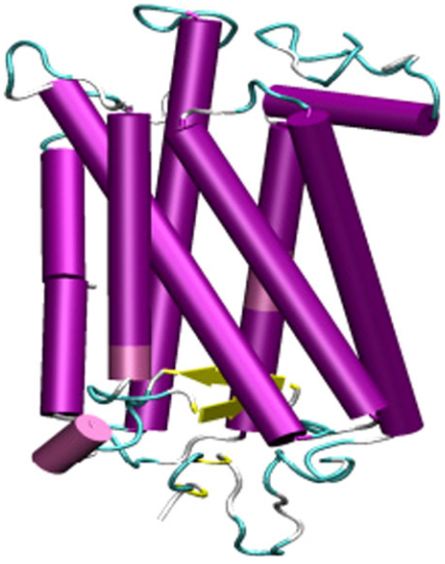

Fig. 3. Crystal structure of ground state rhodopsin from 1U19 [176]. Figure prepared from VMD [177].

Improvements in this structure determination have been reported. The resolution from the three-dimensional crystals was improved to 2.6 Å [119]. A new crystal form was recently reported for rhodopsin at 2.65 Å resolution and the structure was in good agreement with the other crystal structures [120]. These reports were followed by further improvement to 2.2 Å [121]. This higher resolution structure revealed more details about the binding of the retinal and the twisted conformation in the inactive ground state of rhodopsin.

## 10. Three-dimensional structures from sparse constraints

The structural detail for inactive rhodopsin has proven to be exceptionally important to advancing mechanism studies in the field of GPCRs. However, the limitation is that the structure for only one GPCR has been yet solved. With the wide range of cellular functions controlled by GPCRs, structural information for some of the remaining hundreds of GPCRs is very much needed. Therefore an important question is whether there are other ways to obtain three-dimensional structures for GPCRs, based solely on experimental data and not on modeling.

A study published in 2001 suggested the answer to that question was “yes”. In that report, the structure of bacteriorhodopsin was solved, based on experimental data only, without either two-dimensional or three-dimensional crystals [63]. The approach was based in part on the use of sparse constraints. The use of sparse constraints will first be discussed, followed by a discussion of the use of such an approach for GPCRs.

Another such approach using sparse constraints employed a two-step approach to obtaining a structure. First a basis set of 14 crystal structures already reported for integral membrane proteins built around helical bundles was analyzed. Information concerning helix packing was obtained. Second a set of experimental distance constraints was identified from published experiments utilizing a variety of techniques sensitive to distance within the GPCR (these will be described below in more detail). Third a penalty function was developed to drive simulated annealing of a model towards a structure that satisfies all the experimental distance constraints. With this approach, only 27 experimental distance constraints were required to produce a structure for rhodopsin with an RMSD of 3.2 Å relative to rhodopsin for the transmembrane domain (assuming that the transmembrane segments were helices) [122].

Girvin et al. exploited 99 experimental distance constraints from the integral membrane protein, lactose permease, many from the laboratory of Kaback, to produce a structure of the transmembrane domain of this protein [123]. The experimental distance constraints defined inter-helical site-to-site distances in the 12 transmembrane helices that constitute the transmembrane domain of this transporter. The model was subjected to simulated annealing with distance constraints and the resulting structure agreed with the experimental site-to-site distances. This work also assumed that the transmembrane segments were

helical. This report demonstrated that a reasonable structure of the transmembrane region could be defined in this manner.

The structure determination for bacteriorhodopsin noted earlier avoided one of the assumptions of the previously described two studies on rhodopsin and lactose permease. In the bacteriorhodopsin study, the conformation of the transmembrane segments of the protein were defined by solution structure determinations of synthesized peptide fragments using high-resolution NMR techniques. Furthermore, the conformation of the loops connecting the helices were determined by NMR using peptide fragments as well. All the peptide fragments (13) overlapped their neighbors in the sequence in a helical region, so that the fragment structures could be linked by superposition of the overlapping regions. This process defined the full secondary structure of the protein (details of this approach to defining secondary structure were presented in the early part of this review). Then inter-helical experimental distance constraints were introduced and simulated annealing was used to produce a structure that satisfied all the available distance constraints: a limited set of interhelical distances characterizing the packing of the helices, and a much more extensive set of NOE-based distance constraints characterizing all the secondary structure of the protein. The assumption in this approach is that the secondary structure in the fragments is the same as in the intact protein. Direct comparisons with known crystal structures of bacteriorhodopsin confirmed that this assumption was valid. The resulting structure showed an RMSD with respect to the bacteriorhodopsin crystal structures of 2.9 Å for the transmembrane domain [63]. This fit is not far from the RMSD characterizing comparison of one crystal structure with another from different structure determinations of bacteriorhodopsin. This result indicated that valuable structural information could be obtained from sparse distance constraints and secondary structure determination by NMR from peptide fragments of the protein for proteins built around helical bundles (this approach would not be expected to be useful for membrane proteins built on β-structures such as the β-barrels of the porins).

The success of the above studies provided the basis for an alternate structure determination for bovine rhodopsin.

Step 1. Following the pattern with bacteriorhodopsin, a set of overlapping peptide fragments of rhodopsin that spanned the full sequence of the protein was synthesized and their structures determined by high resolution NMR. These peptide fragments formed helices or turns for the most part [16,53,56–58,60,107,124].

Step 2. A set of site-to-site experimental distance constraints was harvested from a rich literature of such experiments for bovine rhodopsin (see below).

Step 3. A starting model was prepared by superimposing the overlaps of the peptide fragments. To this model all the distance constraints from step 1 (NOEs) and step 2 were written on the model. This construct was then subjected to simulated annealing and a structure obtained that satisfied all the distance constraints from step 1 and 2.

The result of this process was a structure for ground state bovine rhodopsin [125]. This structure exhibited an RMSD with the first crystal structure of rhodopsin of 1.85 in the transmembrane region, indicating good agreement. This structure provides complementary information relative to the crystal structure in that the cytoplasmic face of the receptor, which couples with the G protein, is defined in this structure and the cytoplasmic face is partly undefined in the crystal structure. This approach has applicability to structure determinations of other GPCRs and to other integral membrane proteins built around transmembrane helical bundles. The site-to-site distances are obtained from a variety of experimental measurements. The richest set of such distances were obtained through site-directed spin labeling in which dipolar interactions between two spin labels at known sites were interpreted in terms of specific distances [126–143]. Another set of experimental distances can be inferred from disulfide bond formation [144–147]. Yet another distance constraint could be inferred from engineering a metal binding site [148], another from spin label–nuclei dipolar interactions [149] and another from dipolar interactions between fluorine nuclei [150]. In addition in cases where low-resolution projection maps are available for the protein, adequate interhelical distances can be obtained to support this structure determination.

These results were recently exploited to study the structure of the thromboxane A2 receptor. As in the case of rhodopsin described above, the solution structures of peptide fragments containing loops of the receptor were solved by NMR and these structures were assembled into a structure of the whole protein. For the transmembrane region of this protein, a model based on rhodopsin was used [108]. These results suggest that this approach to GPCR structure will be useful into the future, in the absence of readily obtained crystals suitable for X-ray crystallography.

## 11. Activation of rhodopsin

All the structural studies described above provide information about the structure of the inactive form of the GPCR, rhodopsin. The mechanism of activation of a GPCR is of intense interest. As before, the greatest depth of information is for bovine rhodopsin and this will now be discussed.

Rhodopsin, once excited by light, cycles through a series of spectral intermediates that produce metarhodopsin I. During this process as currently understood, nearly all the changes in the receptor are confined to the ligand with few changes in the protein structure. It is only upon moving to metarhodopsin II, the next step after metarhodopsin I that the receptor becomes active, the conformation changing such that the receptor can bind the G protein and activate it. A recent electron crystallographic structure with a predominance of receptors in the meta I form shows that the protein structure changed little from the inactive ground state rhodopsin [151]. The retinal underwent some change including a change in the twist seen in the ground state, but even though the retinal is less twisted in the metarhodopsin-I state [99], the conformation of the β-ionene ring was similar to the conformation seen in the ground state [152]. As the authors of the crystal structure report point out, there is no gradual, step-by-step change in the conformation

leading inexorably towards an activated state. Rather it appears that the action is focused on the retinal through much of the photocycle [97] and by some process yet to be described, the energy confined to the retinal after isomerization is conveyed to the protein concurrent with the conformational change from metarhodopsin I to metarhodopsin II.

This raises the question: what is the conformation of metarhodopsin II? That question needs to be answered before the mechanism of activation of G protein can be addressed. No crystal structure has yet been reported for metarhodopsin II. Light induces damage to the crystals of rhodopsin [116], indicating that light is inducing a conformational change in the protein, but no usable crystals of metarhodopsin II have been reported. However, many experiments have been reported that add individual details to the understanding of what changes when metarhodopsin II forms. Some of those experiments will be recounted here.

The ligand, retinal, changes position in the protein upon activation to metarhodopsin II. The retinal translates in the helical bundle in the direction of TM5 with a concomitant rotation of the C-20 group of retinal [153]. Consequently the contact between C-20 of the retinal and W265, found in the ground state, is lost and a contact of W265 with the C19 methyl group is substituted [105]. The ring of the retinal was reported to contact helix 4 of rhodopsin upon activation, a contact that is not possible in the ground state structure [154].

The powerful approach of site-directed spin labeling provides important site-to-site distances within the helical bundle of the GPCR. From such experiments, investigators have discovered that the helices in the transmembrane domain change their orientation relative to each other when metarhodopsin II is formed. For example, TM2 of rhodopsin moves relative to helix 8 upon activation to metarhodopsin II [155], as does TM6 [141]. The movement of TM6 was described as a rigid body movement [129]. However, studies of the influence of prolines on helix conformation indicated that the helical segments on either side of the proline can swivel with respect to each other and this can be a source of some of the conformational changes seen in the transmembrane domain [156,157]. TM7 and TM1 become further separated [142] upon activation. A caution in the interpretation of these inter-site distances is in order. These site-directed spin label experiments produce approximate distances between sites (with an uncertainty derived from the length of the spin label and its position). When changes in the distances occur, one cannot say with certainty whether one or both of the helices (to which each of the two spin labels are attached) moves; only that the distance between them changes.

Structural changes mediated by membrane phospholipids may also play a role in activation. The region of helix 8 is dependent on membranes: it is helical in the presence of membranes and is non-helical in the absence, as described in the discussion of fragments of rhodopsin. Phosphatidylserine content in the membrane particularly stabilizes the helical form [61]. Many years ago it was reported that phosphatidylserine in the disk membrane has a special relationship with rhodopsin, apparently binding to the receptor [158].

Data such as those derived from site-directed spin labeling, provide some of the few details known about the structural changes that occur in the protein upon activation to metarhodopsin II. Employing the same approach used to successfully determine a structure for ground state rhodopsin from sparse distance constraints, a structure for metarhodopsin II was reported [159]. This structural determination exploited the changes in distances from site-directed spin labeling to define the structural changes that occurred in three dimensions when metarhodopsin II was formed. The success that attended the determination of ground state rhodopsin calls for a closer look at what this structure can report on the conformational change that results from activation of this GPCR.

The greatest density of long-range distance constraints were in the cytoplasmic face of the receptor, so that is the region that should be examined more closely, and is of considerable interest since it is the surface that couples to the G protein. The most obvious change that occurs in the cytoplasmic face of this receptor is the opening of a cleft in the surface. This results from a breaking of the ionic lock between R135 (of the highly conserved D(E)RY sequence at the end of helix 3) and E247 on helix 6. This ionic interaction stabilizes the ground state and apparently must be broken to form the excited state. The cleft that appears is an obvious candidate for interaction with portions of the G protein, as had been suggested previously [160]. Using recent studies on fragments of the G protein binding to metarhodopsin II [65] and the structure derived for metarhodopsin II [159], as well as other experimental clues to the interface, a model for binding of the G protein, transducin, to metarhodopsin II was described [161]. The hypothesis was advanced that the G protein bound through an induced fit mechanism in which the binding energy of transducin to metarhodopsin II was utilized to induce a conformational change in the G protein.

Another report [162] offered a model for the activated state of rhodopsin that also utilized some of the same experimental data as the study described above. The approach was somewhat different, but still employed a simulated annealing to achieve a structure that satisfied the distance constraints characteristic of the metarhodopsin II state. Interestingly this determination began with the crystal structure of the ground state and perturbed that structure with the distance constraints of the excited state. In agreement with the previous structure [159], the salt bridge between R135 and E247 was broken and helix 7 kinks upon activation. As well, a large change in position of helix 8 was noted.

An alternative approach to the definition of the structure of the cytoplasmic face of metarhodopsin II used antibodies that recognized the difference in conformation of the surface between the inactive and activated receptor. Unique antibodies were obtained that stabilized either metarhodopsin I or metarhodopsin II. By identifying the epitopes recognized by these antibodies, the authors concluded that conformational changes occurred in the cytoplasmic face between the two forms of the receptor. In particular, their data suggested changes in the C-terminus of the protein, the third cytoplasmic loop and helix 8 [163]. One of these antibodies (K42-41L) and its epitope were

investigated in detail. K42-41L stabilized metarhodopsin I. The epitope that was recognized by K42-41L likely was not available for binding in metarhodopsin II. Using a peptide (TGALQERSK) that mimicked the binding of the epitope to the antibody, the structure of the peptide bound to the antibody was determined, and, presuming that this conformation reflected the conformation on the surface of the receptor, concluded that the third cytoplasmic loop changed conformation upon activation [164]. This conclusion was consistent with the conformational changes observed in the two structures described above for metarhodopsin II. In this regard, the observation that loops from the cytoplasmic face of rhodopsin have been fused with thioredoxin in a configuration that successfully activated the G protein, transducin, indicated that the loops could form a surface with the binding site for the G protein as well [165]. Apparently there is considerable local stability in some of the loops.

## 12. Activated states of non-rhodopsin GPCRs

Limited information is available on the structures of the excited state of other GPCRs. A few examples will be given, though there is not much detail on any one receptor. Work on the CB1 receptor implicated structural changes in the third cytoplasmic loop [166], parallel to the conclusions for rhodopsin. Echoing what was described above, swivels enabled by prolines were implicated in the conformational changes characterizing the activation of the human prostacyclin receptor [167]. In particular a proline in helix 3 was found to be necessary for the transition to the active state of the receptor. A study on the angiotensin II receptor found an alteration in conformation involving TM2 in a constitutively active receptor [168]. Their data also implicate a change in the interaction between TM2 and TM7 upon activation [169]. Disulfide crosslinking studies have been used to probe conformational changes upon activation of the muscarinic acetylcholine receptor and the data suggested a structural change in the interaction between TM5 and TM6 at the cytoplasmic end as well as an alteration of the helix structure of helix 6 at the end close to the cytoplasmic face [170]. Interestingly, NMR data previously indicated that the cytoplasmic end of TM6 is relatively unstable as a helix [125].

## 13. Unresolved structural issues

Although a great need continues for new structural information of GPCRs, there is an as yet unresolved issue among the published reports that may have importance to the activation process for some GPCRs. Helix 8 is a helix that extends at approximately a right angle from the end of TM7 to the palmitoylation sites in the crystal structure of rhodopsin. This helix has been seen in peptide fragments of other receptors as well [20,31,37,38,42,43,171]. Although many structural changes occur involving helix 8 upon activation, its conformation in metarhodopsin II is as yet uncertain. It has been suggested that a significant conformational change, destabilizing helix 8, may occur upon activation of rhodopsin [21]. This suggestion is supported by studies on peptide fragments of the carboxyl terminus of rhodopsin that show no intrinsic propensity for the helix 8 segment to form a helix spontaneously in solution [172]. Recent molecular dynamics studies show stability of this segment in the presence of a lipid bilayer [173]. It is particularly noteworthy that in both rhodopsin [21,61,174] and in the angiotensin II receptor [171] the structure of this portion of the carboxyl terminus is sensitive to the lipid content of the bilayer, in particular the presence of anionic phospholipids. These data collectively indicate that this portion of the protein may be conformationally flexible depending upon the state of the proteins and may offer a point of conformational modulation influenced by the lipid bilayer.

A second unresolved issue is the conformation of the carboxyl terminus of rhodopsin. FTIR studies suggested the presence of some limited β-structure in the carboxyl terminus [175] and in agreement, studies of the C-terminus by NMR revealed some modest β-structure amongst a reasonably well-ordered fragment of the receptor [16,60]. Yet experiments from site-directed spin labeling in the carboxyl terminus suggested that the carboxyl terminus was largely disordered [132]. The X-ray crystal structures do not have sufficient order in that region of the protein to resolve the issue.

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