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Review

Structural studies on rhodopsin

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Abstract

Bovine rhodopsin is the prototypical G protein coupled receptor (GPCR). It was the first GPCR to be obtained in quantity and studied in detail. It is also the first GPCR for which detailed three dimensional structural information has been obtained. Reviewed here are the experiments leading up to the high resolution structure determination of rhodopsin and the most recent structural information on the activation and stability of this integral membrane protein.

Keywords: Metarhodopsin; Integral membrane protein; G protein coupled receptor

1. Introduction

G protein coupled receptors (GPCR) represent a very large family of integral membrane proteins involved in a wide variety of signal transduction, largely at the plasma membranes of cells. Estimates vary, but as many as 1000 such receptors may exist, controlling vision, olfaction, taste, and many specific cellular functions through hormones and other signals.

Vision is one of the ''senses'' mediated by GPCRs. Two distinct kinds of cells provide the extant sensitivity to the spectrum of visible light of higher animals. Rod cells in the retina are exquisitely sensitive to low levels of light, acting effectively as single photon counters. These cells provide sensitivity to light in black and white. The other major cell type in the retina that is sensitive to light is the cone cell. Cone cells are perhaps 100 times less sensitive to light than rod cells, but provide color vision. In human retinas, three cone subtypes are known, each sensitive to different band widths of visible light.

The retinal rod photoreceptor is a highly polarized, post-mitotic cell which is involved in signal transduction. The rod cells in vertebrate and invertebrate retinas are part of a complex array of neural and epithelial cells that are responsible for the capture of light and the transmission of the resulting nerve impulse to the brain. Rod cells make vision at low light levels possible, and the degeneration of these cells leads to night blindness. These rod cells are divided morphologically into an inner segment and an outer segment. The outer segment of the rod cell encloses a stack of densely packed, closed, flattened membrane sacs referred to as disks, which are stacked along the long dimension of the outer segment. This stack of disks within the rod outer segment (ROS) undergoes dynamic renewal. The disks are formed from evaginations of the ROS plasma membrane at the base of the ROS and progressively move up the outer segment as additional new disks are formed. Old disks at the apical tip of the rod are shed and then phagocytosed by the overlying pigmented epithelium. Thus, the outer segment is in a constant state of degradation and renewal [1,2]. In vertebrates, the transit of disks from the base to the tip of the outer segment requires approximately 10 days.

The ROS membranes contain lipids and proteins, in approximately equal amounts by weight [3,4]. Phospholipids represent almost 90 mol% of the total ROS lipids, while cholesterol accounts for less than 10 mol% on average [3,5]. Cholesterol is not uniformly distributed among the ROS disk membranes, however. Newly formed disks are high in cholesterol content, similar to the plasma membrane from which they form. As the disks age, the cholesterol content decreases, until it reaches 5 mol% or less in the old disks [6,7]. The change in cholesterol content with age of the disk membrane is related to a modulation of activity of rhodopsin in that disk membrane. High cholesterol leads to inhibition of receptor function [8,9].

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Cone cells also contain GPCRs sensitive to light. These GPCRs are found in evaginations of the plasma membrane of the outer segment that resemble the stacked disks of the rod cell except that they are part of a continuous plasma membrane. Interestingly, because the cone rhodopsins are in the plasma membrane, they are in a high cholesterol environment and cholesterol is known to inhibit receptor function. This difference in morphology from the rod cell may explain the much higher sensitivity of the rod cell to light, because in rod disks, separate from the plasma membrane, membrane cholesterol is low and rhodopsin function is greatly enhanced.

Glycerolipid constituents are not constrained to remain with the disks into which they were initially assembled; rather, individual lipid classes have distinct turnover rates which are considerably more rapid than those of the membrane proteins [10–16]. Phospholipid fatty acid composition among disks is also a function of disk age [17].

In striking contrast to the complexity of the ROS lipid molecular species composition [17,18], rhodopsin (the visual pigment) accounts for about 95% of the total ROS membrane protein [19,20]. Rhodopsin, once assembled into a disk, remains associated with that disk throughout its lifetime in the ROS. Thus, the turnover of rhodopsin parallels the basal-to-apical transit time of the disks in the ROS, and the relative location of a disk along the length of the ROS reflects the age of its protein constituents (i.e., the basal-most disks contain the most recently synthesized proteins) [21,22]. The binding of transducin by rhodopsin is also a function of disk age [23].

When light strikes the ROS and is absorbed by the photopigment, rhodopsin goes through a series of spectrally defined intermediates. The transition of metarhodopsin I to metarhodopsin II (Meta II, the active form of the receptor) stimulates the binding of the G protein, transducin. Transducin becomes activated and the α subunit of transducin initiates the cGMP cascade [24] by binding to the phosphodiesterase, and culminating in the hydrolysis of cGMP. Reduction in cGMP levels leads to closure of the plasma membrane Na^+ channels, which results in a hyperpolarization of the plasma membrane that is transmitted to the synapse at the base of the rod cell.

Rhodopsin is a member of a large family of GPCRs. All of these receptors couple to heterotrimeric G proteins as the means to convert an extracellular signal into an intracellular signal. Many of these receptors bind ligands from the cell exterior, which induce a conformational change in the cytoplasmic face of the receptor, enabling binding of the G protein. When the G protein binds to an active receptor, an exchange of GTP for GDP on the α subunit occurs and the α subunit separates from the βγ subunit. In some systems, both the α subunit and the βγ complex can function in signaling. Upon activation, the α subunit of the G protein can modulate the activity of a target protein, often an enzyme. The α subunit is itself a GTPase, and thus with time, the GTP bound to the α subunit is hydrolyzed. The GDP-bound form of the α subunit is inactive and can reassociate with the βγ complex to recycle the G protein.

Rhodopsin is ultimately responsible for the initiation of visual signal transduction. The ligand for rhodopsin is 11-*cis* retinal, which is covalently bound to the receptor in the dark-adapted state. The retinal is photosensitive and confers on the receptor the sensitivity to light. Photoactivation to all-*trans* retinal provides the trigger to a conformational change in the receptor to the active form (Meta II). The all-*trans* retinal is then expelled from the receptor and is cycled, through the adjacent pigmented epithelium, to 11-*cis* retinal, ready to recombine with a bleached opsin (rhodopsin without retinal) to re-form rhodopsin.

Rhodopsin is the most intensively studied member of the GPCR family because it is the only member that is naturally present in high abundance in biological tissues. Earlier reviews have been published (for example, see Ref. [25]). After methods were developed to isolate ROS disk membranes [26], relatively large amounts of natural membrane containing predominantly one membrane protein could be obtained for study. Subsequently, the purification of rhodopsin on an affinity column in detergent was reported [27] and reconstitution of purified rhodopsin into membranes of defined lipid content could be achieved [28,29].

Rhodopsin is also the first (and only) GPCR for which high-resolution three-dimensional structural information is available. A crystal structure of bovine rhodopsin was published in 2000 [30] (R.E. Stenkamp et al., this issue) and a second structure obtained by an alternative approach, was published in 2001 [31]. This review will explore the early literature leading up to the recent structure determinations and will then focus on the most recent structural information which has revealed for the first time the conformational change that occurs upon receptor activation.

2. Early structure results

The first data on the three-dimensional structure of bovine rhodopsin came from circular dichroism (CD) studies of sonicated disks [32] and of the purified protein [33,34]. The CD data were analyzed according to secondary structure content and that analysis was consistent with a structure containing a bundle of seven transmembrane helices [35]. Data also suggested that when light was absorbed by rhodopsin, movement occurred within the bundle of helices. Later, FTIR data also provided information on the helices of rhodopsin [36].

The next step in understanding the three-dimensional structure of bovine rhodopsin was achieved with the publication of the primary sequence [37,38]. The primary sequence is represented in Fig. 1, along with the hydropathy plot. This analysis suggests several hydrophobic segments

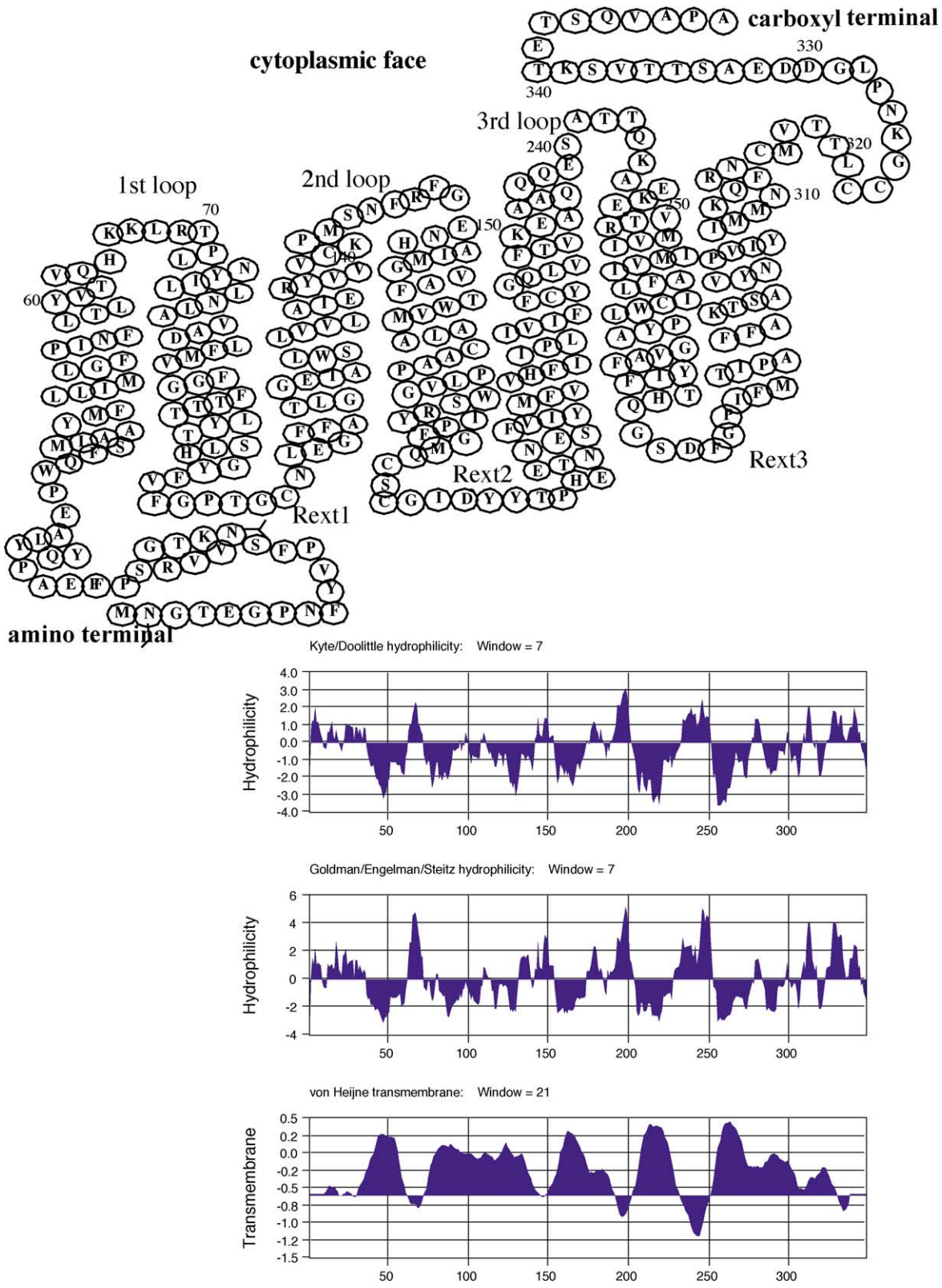

Fig. 1. Top: schematic representation of the primary sequence of rhodopsin. Bottom: hydropathy plots of the sequence of rhodopsin.

in the protein. In particular, six hydrophobic segments are relatively obvious from such a plot, consistent with six of the seven putative transmembrane helices. As is now known, the seventh helix is quite polar in the middle with one lysine , one serine and one threonine, and thus does not show well in the hydropathy analysis.

The next milestone in the development of structural information for rhodopsin came about a decade later with the publication of a low-resolution structural analysis from two-dimensional crystals of bovine rhodopsin [39]. Electron density maps suggested positions for most of the putative transmembrane helices and these helices were assigned using a model developed by Baldwin et al. [40]. Baldwin performed an extensive analysis of the available sequences of GPCRs (with the advent of modern molecular biology, the number of GPCR sequences had increased from one in 1983 to several dozen in the early 1990s). She identified a number of conserved residues in the transmembrane region of the protein. She developed a useful nomenclature of labeling these residues and some suggested rules for the likely disposition of such conserved residues within the structure.

This was followed by a low-resolution three-dimensional structure for bovine rhodopsin in which, for the first time, one could see the bundle of transmembrane helices and trace most of them through the electron density. One of the first observations from such analysis was that, contrary to many expectations, the structure of bovine rhodopsin was not the same as bacteriorhodopsin [41]. The packing of the helices differed between the two photosensitive proteins. This was made more obvious by studies on frog rhodopsin [42] and squid rhodopsin [43], which showed strong similarity with bovine rhodopsin and significant differences from bacteriorhodopsin. These observations called into question some of the early models for bovine rhodopsin built on the template of bacteriorhodopsin. However, more recent models using some limited experimental data and the models and principles enunciated by Baldwin proved to be more accurate [44].

As details emerged about the packing of the helices in rhodopsin, several distinctive features were identified. One feature is the tilt of helix 3. Helix 3 is the most severely tilted helix of the bundle in the dark-adapted state structure of rhodopsin. At the other extreme, helices 4, 6 and 7 were suggested to be the closest to perpendicular to the membrane surface. These features have survived into the most recent three-dimensional structure determinations for rhodopsin, as will be seen below.

3. Studies on proximity of sites within the protein

Several different methods were developed in the 1990s to measure distances between discrete sites on the protein, or to probe close contacts within the rhodopsin structure. Many of these studies employed one of two different methods. One method is site-directed spin labeling, developed as a collaboration between the laboratories of Hubbell and Khorana for use on bovine rhodopsin. The other, described in detail by Oprian et al., explores close contacts between helices with engineered disulfide bonds.

Site-directed spin labeling has proven a powerful method to obtain long-range distances between specific sites in the intact protein [45]. Using mutagenesis, one can control the availability of cysteines for reaction with spin label probes. For example, one can make a cysteine-less protein and then reintroduce two cysteines at specific positions. Labeling this protein with spin labels produces two labeled sites. The dipolar interaction between the two sites can be measured and since the dipolar interaction is distance-dependent, the magnitude of that dipolar interaction can be interpreted in terms of a distance in angstroms between the labels. Distances between about 5 and 25 Å can be determined by this method.

The laboratories of Hubbell and of Khorana have collaborated to create a wealth of distance data for rhodopsin using this technique [46–61]. These experiments have both provided data on distances between specific sites in dark-adapted rhodopsin and also have provided data on the transient state of metarhodopsin II, the activated state of rhodopsin. From these data, they concluded that movements in helix 3 and helix 6 are among the conformational changes that occur when rhodopsin converts to metarhodopsin II. Some of the distance information obtained for rhodopsin by this technique is summarized in Table 1.

The spin label experiments also provide indirect information on secondary structure. Periodicities in the EPR spectra from labels at sequential positions in the sequence point to the occurrence of α helix, in some cases. With this kind of experiment, they were able to locate the carboxyl end of helix 5, for example [48]. These experiments also reveal information about the exposure of sites to the aqueous phase and about dynamics of the polypeptide chain at the spin label position.

Engineered disulfide bonds have provided considerable information about contacts between helices in the transmembrane helical bundle of rhodopsin. Much work in this area has come from Yu et al. [62]. Putative contact points

Table 1
Experimental long-range constraints for rhodopsin

	R* (Å)	R (Å)
V139C_K248C	23–25	12–14
V139C_E249C	15–20	15–20
V139C_V250C	12–14	15–20
V139C_T251C	23–25	12–14
V139C_R252C	23–25	15–20
H65C_C31 [49,50,168]	12–15	7–10
C140_S338 [169]	15–21	
V204C_F276C [62]	2–5	2–5
I251_V138 [68]	<13	
C140_C222 [67]	>7	2–5
C140_Q225C	2–5	2–5
K245C_Q312C	>7	2–5
R135C_V250C	>7	2–5
K245C_S338C [56]	2–5	2–5
S338C_T242C [53]	>7	2–5
Y136C_C222	>7	2–5
Y136C_Q225C	2–5	2–5
Interhelical distances [40]	X	
Helix assignment of Baldwin [145]		

Where R* is Meta II, and R is rhodopsin (not activated).

are probed by introducing cysteines at key positions in the protein and looking for the formation of disulfide bonds between those two cysteines. This approach has produced a number of contact points within rhodopsin and some of these data are summarized in Table 1 [63–65].

These experiments have yielded important information on the conformational change from dark-adapted rhodopsin to metarhodopsin II. In some cases, the disulfide bonds inhibit the change in conformation suggesting that those contact points are no longer in contact in metarhodopsin II. In other cases, the presence of the disulfide bonds does not inhibit the formation of metarhodopsin II, and thus indicated that those points of contact are present in both the dark-adapted state and the active state of the receptor [66,67].

The success of these disulfide experiments depended upon another important observation about the structure of rhodopsin. Oprian et al. were able to express rhodopsin in two pieces, each piece containing two or more transmembrane helices. For example, one of the two pieces contained four of the transmembrane helices of rhodopsin and the corresponding linking loops, and the other piece contained the remaining three of the transmembrane helices and the connecting links. These researchers noted that the separate, co-expression of these pieces of rhodopsin led to the formation of pigment; that is a protein that incorporated the ligand, retinal, even though the two pieces were not covalently linked. By some process after expression, the two pieces came together in the membrane in a native-like conformation. This work suggests considerable stability within the helical bundle that will be discussed later in this article.

If a metal ion binding site can be engineered between two or more helices with residues from each of the helices contributing ligands to the metal ion, then one can identify a close approach of those helices. Such an experiment was performed in rhodopsin, which showed contact between helices 3 and 6. Furthermore, this contact was apparently broken upon activation of the receptor [68].

4. Retinal in rhodopsin

The ligand, retinal, has been studied extensively in rhodopsin. It is beyond this review to consider the whole of this body of work. However, among this important body of work are structural studies of the retinal in the protein [69–78]. These studies suggested interactions between specific amino acids in the rhodopsin structure and the retinal chromophore, demonstrated the protonated Schiff base the retinal forms with lys296, and provided detailed information on the orientation and location of the retinal in rhodopsin.

5. Three-dimensional structure of rhodopsin

The X-ray crystal structure of rhodopsin [30] is described in detail elsewhere in this volume. However, the importance of this contribution should be emphasized. This is the first three-dimensional structure of any member of the GPCR family. It was possible to see for the first time details of the organization of the transmembrane helical bundle, the location of retinal in the structure, the intradiskal (extracellular) face, the palmitoylation of the protein, and the closure of the intradiskal face over the retinal, locking it into the structure of the protein. Missing from this structure are details of the cytoplasmic face of the protein.

Several years before the publication of this structure, another approach to the structure of rhodopsin was begun [79]. This approach, to be described below, ultimately led to the publication of a second structure of rhodopsin, in excellent agreement with the crystal structure, but providing greater detail in the cytoplasmic face of the protein. A somewhat simpler version of this method has been used to determine, in part, the backbone fold for other proteins [80–82]. This approach circumvents the need to have crystals and is applicable to structure determination for other GPCRs and perhaps other membrane proteins as well.

A growing body of data suggests that solution structures of peptides derived from some classes of proteins retain the secondary structure of the parent protein because of the dominance in α-helices and turns of short-range interactions [83] that can be captured in peptides. Studies on segments of soluble proteins forming α-helices show that peptides containing these sequences form α-helix in almost every case under some solution conditions [84–93]. Peptides representing segments that are turns in the native protein also show turns as peptides in solution [89,92,94–100]. In some cases, the entire sequence of a helical bundle protein has been incorporated in a series of peptides spanning that sequence and the individual peptides have reported the secondary structure of much of the native protein with fidelity [97,101–104].

The use of peptides to determine the secondary structure of membrane proteins is becoming widely accepted. Recent studies on transmembrane proteins, including bacteriorhodopsin, rhodopsin, tachykinin receptor, PTH receptor, angiotensin II receptor, α-factor receptor, potassium channels, and glycophorin, showed that peptides from transmembrane helices formed α-helices independently from the rest of the protein [93,105–115] and peptides from turns show turns independently of the remainder of the protein [116–122].

The issue of independent stabilization of membrane protein domains has been investigated. For example, glycophorin is a membrane protein that can be cleaved into fragments that retain the secondary structure of the native protein after separation [123]. Both rhodopsin [35] (as described earlier) and the Na^+ K^+ ATPase [124] are membrane proteins for which proteolytic fragments retain structures characteristic of the native protein. Yu et al. [62] have shown that rhodopsin can be expressed as fragments that spontaneously assemble after expression

into a functional unit in the membrane. Similar studies have been done on the α-factor receptor [125]. The cytoplasmic loops of rhodopsin have functions separate from the remainder of the protein [121,126,127], and have structure as well [79,119].

It can therefore be hypothesized that the intrinsic structures of peptides containing the amino acid sequences for turns or for transmembrane helices of membrane proteins built of helical bundles will be the same that those sequences adopt in the native protein. This hypothesis could provide access to important structural information for a transmembrane protein that may be available from no other approach.

6. Test of alternative approach to membrane protein structure: bacteriorhodopsin

This hypothesis was tested for membrane proteins using bacteriorhodopsin, a protein whose structure was already known. The structure of bacteriorhodopsin consists of a bundle of seven transmembrane helices connected by turns. Hunt et al. [93] have found that the transmembrane helices of this protein are independently stable folding units and thus can be considered protein domains [105]. Several X-ray crystal structures are available for this membrane protein [128–131].

A series of peptides were designed for bacteriorhodopsin [132]. Each peptide was designed to encompass either a transmembrane helix of the protein or a turn. Peptides containing a turn also included some of the helices (one to two turns) that connect to the turn on both sides. Furthermore, each peptide was designed to overlap each of its neighbors in the series by 10 amino acids. The overlap was necessary because structures of peptides in solution typically show disordered termini. To obtain structural information on the entire sequence thus necessitated a design in which the disordered ends of each of the peptides could be ignored.

The structures of these peptides in solution were determined by two-dimensional homonuclear ^{1}H NMR as described in detail previously [117,132]. Most of the peptides in this set from bacteriorhodopsin were hydrophobic and not soluble in water. All peptides in the series were soluble in DMSO except for the peptide corresponding to helix G, which was not soluble in DMSO or in chloroform/methanol solutions. Previous work had shown that helix G was not stable in detergent micelles either [93]. DMSO was chosen as a solvent that could be used in common for the 12 of the 13 peptides that were soluble.

The solution structure of each soluble peptide was determined using ^{1}H nuclear magnetic resonance (NMR). Each of the 12 peptides exhibited one family of structures in solution. Peptides corresponding to helices A, B, C, D, E, and F of bacteriorhodopsin formed helices in solution that agreed well with the crystal structure [132]. The peptides corresponding to all six turns from bacteriorhodopsin form turns with the same residues in both the crystal structure and the peptide [132]. Most of these peptide structures superimpose on the crystal structure with an rmsd less than 2.5, indicating that the structures in the peptides are the same as in the crystal structure (given the limited number of constraints normally available from modest-sized peptides in solution) [132]. For comparison, the overall backbone rmsd between two crystal structures of bacteriorhodopsin (2BRD and 1AP9) is about 2.3. High B factors from the loops in the crystal structures complicate comparisons; nevertheless, the residues that define the turns in the crystal structures and in the peptides are the same.

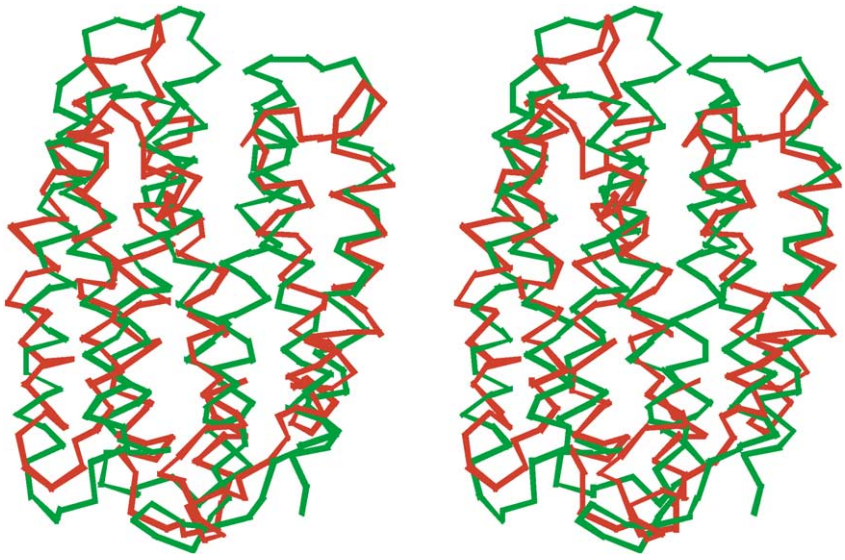

Fig. 2. Structure of bacteriorhodopsin obtained as described in the text and in Ref. [132]. The structure obtained from the NMR data is in red (1 lom) and the structure from X-ray crystallography (2brd) is in green.

Exploiting the overlap of adjacent peptides, a continuous construct of all the peptides can be made by superimposing the backbone atoms of the overlapping regions. All available experimental distance constraints were then written on this construct as described below, including 2438 distance range constraints and 75 angle constraints. Some interhelical distance constraints were used in this structure determination to help in organizing the helical bundle. These constraints can be obtained from more than one source: low-resolution diffraction studies of two-dimensional crystals, solid state NMR experiments (rotational resonance), cysteine scanning (disulfide bond formation), dipolar interactions between spin labels, fluorescence energy transfer measurements, engineering of metal binding sites, or complementary mutagenesis experiments.

Simulated annealing is used to optimize the conformation of the protein with respect to all the experimental constraints simultaneously. The result is shown in Fig. 2 [132]. Fig. 2 also shows an overlay of this structure on a previously determined structure of bacteriorhodopsin. This comparison shows considerable agreement between the two structures determined by different methods (rmsd 2.9).

These results suggest that considerable structural information can be obtained from the segmented approach described in this work for transmembrane proteins built around helical bundles. This method does not replace X-ray crystallography, but is expected to be useful in the absence of X-ray crystallography.

7. Structure of dark-adapted bovine rhodopsin by NMR

The successful test of this novel approach to structure on bacteriorhodopsin encouraged the application of the approach to a new membrane protein, bovine rhodopsin. As stated earlier, the recent X-ray crystal structure of rhodopsin [30] provided a high-resolution structure of the transmembrane domain of the dark-adapted (not activated) state of this receptor, but did not provide the structure of the cytoplasmic face which couples to the G protein in signal transduction. By applying the novel approach described here to rhodopsin, new information on the structure of the cytoplasmic face of this receptor was obtained and it was possible to map, at least in part, the G protein binding site on this structure.

A series of overlapping peptides spanning the rhodopsin sequence was synthesized. Each peptide was designed to represent either a transmembrane helix of the protein or a turn. Each peptide was designed to overlap each of its neighbors in the series because structures of peptides in solution typically show disordered termini. To obtain secondary structure from the entire sequence thus necessitated a design in which the disordered ends of each of the peptides could be ignored. Solution structures for all the peptides were determined by two-dimensional homonuclear ^{1}H NMR as described in part previously [79,119,133]. Structures were determined in water or in DMSO for water-insoluble peptides (as described above, structures of the water-insoluble helices and loops of bacteriorhodopsin in DMSO superimposed well

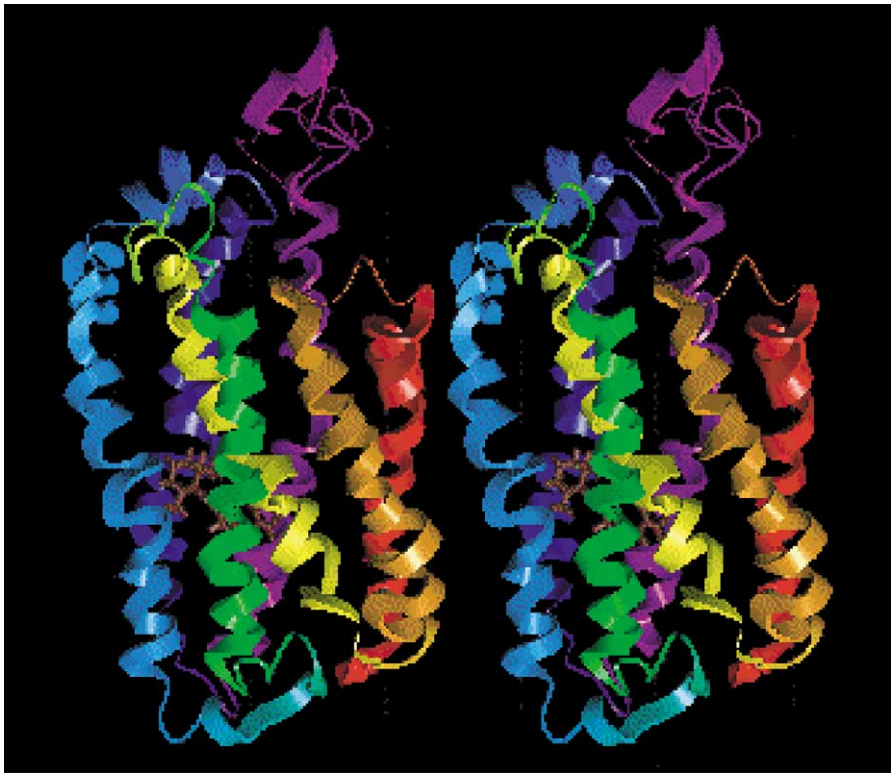

Fig. 3. Three-dimensional structure of rhodopsin in stereo, obtained from NMR data as described in the text (1jfp).

on the crystal structure). Most peptides show well-defined structure except at the extreme termini. Peptides from helices showed helices in agreement with the X-ray crystal structure of rhodopsin [111,112,134]. For most of the overlapping sequences, both peptides were helical in the overlapping sequence. Superposition of those helical overlaps [112] linked the segments into a construct corresponding to the entire sequence of rhodopsin. To K296 11-*cis* retinal was added.

Experimental distance constraints were written into the mol2 file for this construct in SYBYL (Tripos). 3030 short-range NOE-derived distance constraints were available from the NMR structure determinations on the individual peptides. Hydrogen bonds were added where they were observed in the peptide structures. Long-range constraints (see Table 1) from independent experiments on intact dark-adapted rhodopsin were added. The 11-*cis* retinal was constrained by the solid state NMR data of Grobner et al. [76]. The construct with the distance constraints was subjected to simulated annealing (1000 fs at 1000 K followed by 1500 fs cooling to 200 K). As a refinement process, additional hydrogen bonds were added to the constraints when helices became distorted from the original peptide structures by simulated annealing of the whole construct, and additional cycles of simulated annealing were subsequently performed.

The result is a compact structure, strictly from experimental data (no modeling), showing a bundle of seven helices connected by six turns (Fig. 3) [31]. Superposition of this structure on the previously published crystal structure of rhodopsin [30] shows good agreement with the crystal structure in the transmembrane region.

This structure is consistent with information from other experiments for dark-adapted rhodopsin (that were not used in the structure determination). CD data suggested that 60% of the sequence was helical, in good agreement with 64% in this structure [35]. Indirect measurements with spin labels on the intact protein predicted the termination of helix 5 within one residue of that found in this structure [48]. The inferred termination of the seventh transmembrane helix from similar measurements [135] agrees within one residue with this structure. The short anti-parallel β-strands in the carboxyl terminus of this structure agree with FTIR data on the intact protein [36]. Specific interactions between residues 338 and 242 indicate defined structure in the carboxyl terminus [53]. The third cytoplasmic loop projects towards the carboxyl terminus in agreement with AFM data [136].

One significant difference between the crystal structure and the structure derived from the NMR data is found in the carboxyl terminus. In the former structure, the region of residues 311–321 is in an α-helix called helix 8 [30]. In the latter structure, this region is a loop, indicating that there is little intrinsic tendency (from the primary sequence) to form a helix in this region. Therefore in rhodopsin the formation of the helix may well be dependent on an interface; i.e., the lipid bilayer. These observations could be important to structure and function in this protein.

8. Structure of metarhodopsin II

The structure just described is the structure of this receptor in the dark-adapted state. To understand how this receptor system operates, information on the structure of the active state is required. Of the several intermediates in the photocycle, metarhodopsin II (Meta II) is the intermediate which is responsible for binding and activating the G protein, transducin. Therefore, the structure of this intermediate is critical to understanding the mechanism of signal transduction with this receptor system.

Meta II is a transient species. However, it is possible to trap this species by lowering the temperature. With this approach, Hubbell and Khorana were able to use site-directed spin labeling to obtain distance information between specific sites in the protein while it was in the Meta II state. These data are analogous to the data used (see above) to build the tertiary structure of dark-adapted rhodopsin. Oprian et al. have found that some of their engineered disulfide bonds do not inhibit the transition from dark-adapted rhodopsin to Meta II, suggesting that the close contacts, implied by the disulfide bond formation, are preserved in the active state. These data provide additional distance constraints for the active state. These long-range distance constraints include a contact between the β-ionone ring of the retinal and A169 of helix 4 [75].

The structure of the dark-adapted state of rhodopsin was used as the starting point for this structure determination of Meta II. All the long-range interactions specific for the dark-adapted structure were removed and the long-range interactions specific for Meta II were added. Simulated annealing was once again used to fold a structure consistent with the new data set of experimental long-range distance constraints. The resulting medium-resolution structure of Meta II is shown in Fig. 4A. More experimental long-range distance constraints are available for the cytoplasmic face than for other parts of the structure. Therefore, the organization of this region is better defined than other regions of the protein.

Significant structural changes occur upon conversion of dark-adapted rhodopsin to Meta II. The second and third cytoplasmic loops move apart and change their conformation. Upon formation of Meta II, a basic groove opens in the cytoplasmic face of the receptor, exposing a surface that is occluded in the dark-adapted state. Exposure of this new basic surface is likely the signal for G protein binding, since the face of transducin that binds to rhodopsin is acidic [137]. The precise surface for docking is not known. However, the likely binding site for transducin can now be mapped on the cytoplasmic face of Meta II using sequences of peptides that inhibit the interaction between the receptor and transducin [126,138,139]. This mapping suggests contact between the G protein and the groove on the surface of the receptor that is opened upon the formation of Meta II. The most specific recent work is that reporting chemical cross-links between sites on the receptor and sites on transducin that occur in the complex between transducin and Meta II [140,141]. These

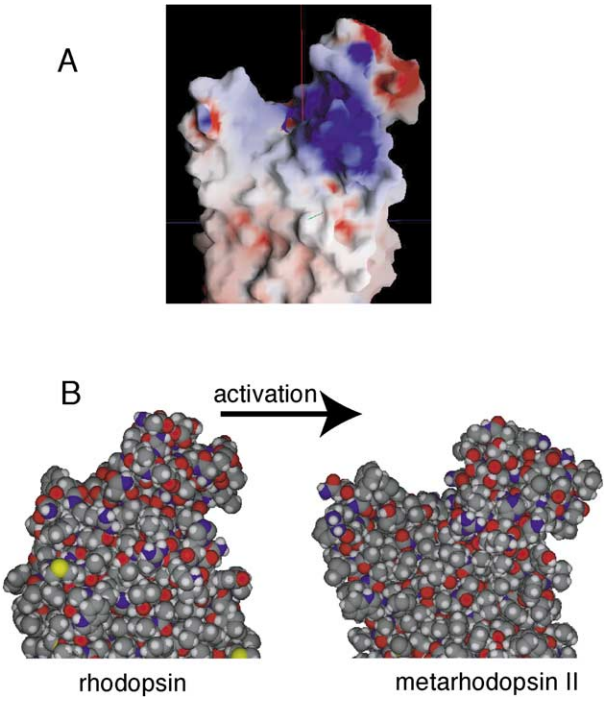

Fig. 4. (A) GRASP picture of the cytoplasmic face of metarhodopsin II (1ln6) [170]. The structure was determined as described in the text. The blue color represents regions of positive charge, and the red color represents regions of negative charge. (B) Comparison of the structure of dark-adapted rhodopsin with light-activated rhodopsin (metarhodopsin II), showing the opening of the surface of the receptor upon activation (see text). The atoms are colored by type.

studies show that in the complex, the N- and C-termini of transducin α are near the third cytoplasmic loop of Meta II. These cross-linking data and the structure of Meta II suggest that the acidic portion of the amino terminal helix of transducin α (see GRASP picture of transducin Ref. [137]) may lie in the basic groove on the receptor when the G protein is bound to Meta II.

The structure of Meta II explains some previously published experiments that concern the cytoplasmic face of this receptor. First, the alteration in cytoplasmic loops 2 and 3 may have been detected previously. These loops contain β-turns [119] and previous FTIR data suggests a change in β-turns in the conformation change from rhodopsin to metarhodopsin II [142]. Second, both the crystal structure and the NMR structure of rhodopsin [30,31] show interactions between R135 and E134 and E247. The structure of Meta II shows that upon activation of rhodopsin, the interaction between R135 and E247 is broken due to the conformational change. This disruption of interactions between R135 and E247 was predicted previously for the corresponding residues in other receptors in the same GPCR family [143,144].

The transmembrane region is defined by fewer experimental long-range distance constraints than are available for the cytoplasmic face. However, the arrangement of much of the helical bundle is locked by recent experimental data [66]. These data indicate that the arrangement of helices 1, 2, 5 and 7 is not changed upon conversion of rhodopsin to Meta II. However, helices 3, 4 and 6 move relative to their positions in the rhodopsin structure, and alter in particular the positions of the helices in the cytoplasmic face of the receptor. Specifically, during the photoactivation and the *cis–trans* isomerization of retinal chromophore, helix 3 is forced outwards probably as a result of the steric interaction between C9 methyl group of the chromophore and residues on helix 3. Within the constraints imposed by the work of Struthers et al. [66], an inward movement of helix 4 is necessary to counteract the outward movement of helix 3. During this cooperative rearrangement, helix 4 is rotated, with A169 coming in close range to the retinal β-ionone ring. The model of Baldwin [145] predicts that helix 4 would be capable of rotation since it does not have a side restricted to interaction with other members of the helical bundle by the polarity.

This helical arrangement is consistent with predictions from previous work. For example, the movement of helices 3 and 4 allow a contact in Meta II between the β-ionone ring of the retinal and A169 of helix 4 as described previously [75]. Research conducted with ring-constrained retinal analogs suggests that the retinal undergoes a twist when photoisomerization takes place, which would force a change in orientation of the β-ionone ring [77]. The β-ionone ring contacts helix 3, and thus a change in the orientation of this ring could induce an alteration in the organization of the transmembrane helices. Those authors suggest an initial movement of helix 3. Such a movement was predicted previously to disrupt restraining interactions that stabilize the inactive state of another receptor in the same family [146]. Helix 6 also moves in this structure of Meta II compared to dark-adapted rhodopsin, and the distance between the cytoplasmic ends of helix 3 and helix 6 increases, as suggested previously [49,147]. Consequently, the cross-sectional area of the molecule on the cytoplasmic side increases modestly in the structure of Meta II compared to dark-adapted rhodopsin, consistent with an expansion of the receptor upon activation [148].

9. Rhodopsin stability

The structures of integral membrane proteins must accommodate interactions with two different environments, the hydrocarbon core of the lipid bilayer in which they are imbedded and the aqueous phase into which they protrude. There is a paucity of studies on the stability of membrane proteins. However, the protein regions that interact with the aqueous phase seem to behave in a manner similar to soluble proteins while those within the hydrophobic core are more structurally stable [149,150]. For example, the two CD transitions exhibited by cytochrome b_5 were interpreted to represent the aqueous and transmembrane domains [151]. For membrane proteins that traverse the bilayer multiple times, the relative importance of the extramembraneous regions and intramembraneous regions to overall protein stability poses an interesting problem. The complexity of

this was demonstrated for bacteriorhodopsin. As stated earlier, some of the helices of bacteriorhodopsin will associate properly without covalently linked loop regions [152,153]. This suggests that the loops may not be important in the stability. However, based on thermal denaturation studies, it was calculated that loss of all loops would lead to instability of bacteriorhodopsin at room temperature [152].

The thermal denaturation of rhodopsin is likely to involve multiple steps including isomerization of 11-*cis* retinal, unfolding of regions exposed to the aqueous media and/or reorientation of the helices. Because opsin does not have 11-*cis* retinal, its denaturation would only involve the later two. Differential scanning calorimetry (DSC) has proven to be a useful tool in investigating the thermal stability of rhodopsin in native disk membranes [154–157] and in disk membranes with altered lipid compositions [158,159]. Interpretation of these studies is complicated by the irreversible nature of the denaturation. However, they showed that both the chromophore and the lipid bilayer effect the transition temperature of rhodopsin denaturation.

Investigations of the scan rate dependence of the calorimetric transition temperature indicated that at the transition temperature there is significant formation of the final, irreversibly denatured species [154,157]. Thus the formation of irreversibly denatured rhodopsin is rapid in comparison to reversible processes. The observed asymmetry of the calorimetric transition is also consistent with this interpretation. The denaturation process of rhodopsin is therefore subject to kinetic constraints. These constraints may also be important factors in the stability of cytochrome *c* oxidase [160], bacteriorhodopsin [161] and the GLUT 1 receptor [162]. These studies suggest that rhodopsin may have a finite, kinetically determined lifetime as a native protein in the membrane. This observation may be important in relation to the transit time of disks in the ROS from the point of biogenesis at the base of the outer segment to the apical tip of the outer segment.

DSC was also used to investigate the relationship of rhodopsin cytoplasmic loops to stability and to begin to relate structural features with rhodopsin stability [157]. Studies of the papain and chymotrypsin proteolytic fragments (loops 3, and both 2 and 3 cleaved, respectively), showed that in the presence of 11-*cis* retinal, the interaction of the helices is sufficiently strong such that the complex denatures as a single unit even though somewhat less energy is required. These studies were also consistent with the involvement of a conformational change in the third loop during the transition to the denatured state. Unlike the case for the unbleached samples or for the bleached papain fragments, the thermal denaturation of bleached chymotrypsin fragments produced two calorimetric transitions. This suggests that the second cytoplasmic loop may be involved in maintaining the relationship between helix 3 and helix 4 in the bundle of helices, and thus their interactions with other helices of the bundle. When loop 2 is proteolyzed and 11-*cis* retinal is not exerting a stabilizing interaction with the adjacent helices, the appropriate orientation of helices 3 and 4 may be lost. This could reduce the helix–helix interactions sufficiently to enable two sets of helices to denature independently.

10. Lipid–rhodopsin interactions in the disk membrane

Rhodopsin is an integral membrane protein, with much of its mass buried in the lipid bilayer. Therefore, the interactions between the lipid and the protein may be important to protein structure and function. This subject requires considerably more investigation. Nevertheless, some structural studies have been reported on the interaction of the lipid component with rhodopsin in the disk membrane. ^{31}P NMR experiments have revealed that in the disk membrane about 15–20 of the phospholipids are interacting significantly with the protein, perhaps binding to sites on the protein [163]. Chemical labeling experiments have shown that some of the phosphatidylserine in the membrane is protected by protein from labeling [164]. Some of the phospholipid in the membrane is protected from phospholipase action; that protected phospholipid component is enriched in phosphatidylserine [165]. These studies collectively suggest that a subset of the phospholipids of the membrane may be binding to rhodopsin and perhaps modulating its structure and function. Therefore, future studies should examine in more detail where on rhodopsin such lipid–protein interactions might have impact.

Biochemical studies have examined in some detail the influence of the lipid environment on rhodopsin function. Membrane cholesterol significantly inhibits rhodopsin function [8,9]. Highly unsaturated phospholipids enhance the activation of rhodopsin to metarhodopsin II [166]. It has now been clearly shown that this modulation is propagated through the structure and dynamics of the lipid bilayer. Recent studies suggest that polyunsaturated phospholipids partition directly into the lipid–protein interface, preferentially to other phospholipid species in the disk membrane [159]. Other studies have suggested that one molecule of cholesterol may also bind to the surface of rhodopsin within the membrane at a site with structural specificity for cholesterol [167].

With the new structural information, these studies on lipid–protein interactions need to be expanded. Questions of how and where the lipids interact with the protein, and in particular, what effects the various lipid components may have on protein structure, function and stability are now very important questions that need to be addressed.

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