Rhodopsin: Structural Basis of Molecular Physiology

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Menon, Santosh T., May Han, and Thomas P. Sakmar. Rhodopsin: Structural Basis of Molecular Physiology. Physiol Rev 81: 1659 –1688, 2001.—The crystal structure of rod cell visual pigment rhodopsin was recently solved at 2.8-Å resolution. A critical evaluation of a decade of structure-function studies is now possible. It is also possible to begin to explain the structural basis for several unique physiological properties of the vertebrate visual system, including extremely low dark noise levels as well as high gain and color detection. The ligand-binding pocket of rhodopsin is remarkably compact, and several apparent chromophore-protein interactions were not predicted from extensive mutagenesis or spectroscopic studies. The transmembrane helices are interrupted or kinked at multiple sites. An extensive network of interhelical interactions stabilizes the ground state of the receptor. The helix movement model of receptor activation, which might apply to all G protein-coupled receptors (GPCRs) of the rhodopsin family, is supported by several structural elements that suggest how light-induced conformational changes in the ligand-binding pocket are transmitted to the cytoplasmic surface. The cytoplasmic domain of the receptor is remarkable for a carboxy-terminal helical domain extending from the seventh transmembrane segment parallel to the bilayer surface. Thus the cytoplasmic surface appears to be approximately the right size to bind to the transducin heterotrimer in a one-to-one complex. Future high-resolution structural studies of rhodopsin and other GPCRs will form a basis to elucidate the detailed molecular mechanism of GPCR-mediated signal transduction.

I. INTRODUCTION TO RHODOPSIN: A PROTOTYPICAL G PROTEIN-COUPLED RECEPTOR

Rhodopsin (Rho) is a highly specialized G protein-coupled receptor (GPCR) that detects photons in the rod photoreceptor cell. Within the superfamily of GPCRs that couple to heterotrimeric G proteins, Rho defines the so-called class A GPCRs, which share primary structural homology (221, 232). Bovine Rho has been the most extensively studied GPCR. A large amount of pigment (0.5–1.0 mg) can be obtained from a single bovine retina by sucrose density gradient centrifugation preparation of the rod outer segment disk membranes (199). The pigment is stable enough in the dark that it can be further purified by various chromatographic procedures, and it remains stable in solubilized form in a variety of detergents (187). Bovine Rho was the first GPCR to be sequenced by amino acid sequencing (93, 193), the first to be cloned (174, 175), the first to be crystallized (186), and the first to yield a crystal structure (194).

Visual pigments share a number of structural features (222). Like all GPCRs, they consist of seven transmembrane segments (H1 to H7). A Lys residue that acts as the linkage site for the chromophore is conserved within H7 in all pigments, and a carboxylic acid residue that serves as the
counterion to the protonated, positively charged Schiff base is conserved within H3. The position analogous to the Schiff base counterion is one helix turn away from the position of an Asp residue conserved in biogenic amine receptors that serves as the counterion to the cationic amine ligands. A pair of highly conserved Cys residues is found on the extracellular surface of the receptor and forms a disulfide bond. A Glu(Asp)/Arg/Tyr(Trp) tripeptide sequence is found at the cytoplasmic border of H3. This sequence is conserved in class A GPCRs and has been shown to be involved in G protein interaction (68, 69). Sites of light-dependent phosphorylation at Ser and Thr residues are found at the carboxy-terminal tail of most visual pigments. These sites are analogous to phosphorylation sites found on the carboxy-terminal tails of other GPCRs (20).

Although it shares many similarities with other GPCR types, as a visual pigment Rho displays many specialized features not found in other GPCRs. In particular, visual pigments are made up of opsin apoprotein plus chromophore. The chromophore is not a ligand in the classical sense, since it is linked covalently via a protonated Schiff base bond to a specific Lys residue in the membrane-embedded domain of the protein (Fig. 1). The chromophore in all Rhos is derived from the aldehyde of vitamin A1, 11-cis-retinal (RET). Some fishes, amphibians, reptiles, and aquatic mammals may also employ the aldehyde of vitamin A2, 11-cis-3,4-didehydroretinal, which contains an additional carbon-carbon double bond. All pigments with a vitamin A2-derived chromophore are called porphyropsins. An important structural feature of the RET chromophore in Rho, in addition to its Schiff base linkage, is its extended polyene structure, which accounts for its visible absorption properties and allows for resonance structures (206).

**FIG. 1.** A secondary structure diagram of bovine Rho. Amino acid residues are depicted in single-letter code. The amino-terminal tail and extracellular domain is toward the top, and the carboxy-terminal tail and cytoplasmic domain is toward the bottom. Transmembrane α-helical segments (H1 to H7) and the cationic amphipathic helix H8 are shown in colored cylinders. An essential disulfide bond links Cys-110 and Cys-187. Cys-322 and Cys-323 are palmitoylated. **Inset:** structure of the 11-cis-retinylidene (RET) chromophore. Carbon atoms are numbered 1 through 20.
Rho displays a broad visible absorption maximum ($\lambda_{\text{max}}$) at $\sim$500 nm (Fig. 2). Photon capture leading to photoisomerization of the 11-cis- to all-trans-form of the RET chromophore is the primary event in visual signal transduction, and it is the only light-dependent step (252). After photoisomerization, the pigment decays thermally to metarhodopsin II (meta-II) with a $\lambda_{\text{max}}$ value of 380 nm. The meta-II intermediate is characterized by a deprotonated Schiff base chromophore linkage. Meta-II is the active form of the receptor ($R^*$), which catalyzes guanine nucleotide exchange by the rod cell heterotrimeric G protein transducin ($G_t$). In contrast to vertebrate vision, invertebrate vision is generally photochromic; a photoactivated invertebrate pigment can be inactivated by absorption of a second photon that induces isomerization to the ground-state cis-conformation.

In the case of the vertebrate visual system, $G_t$ activation leads to the activation of a cGMP phosphodiesterase (cGMP PDE) and the closing of cGMP-gated cation channels in the plasma membrane of the rod cell. Light causes a graded hyperpolarization of the photoreceptor cell. The amplification, modulation, and regulation of the light response is of great physiological importance and has been discussed in detail elsewhere (17, 36, 236, 253). However, it should be pointed out that despite the fact that the visual system functions over about a 10^6-fold range of light intensity, the retinal rod cell has single photon detection capability due to extremely low levels of dark noise in Rho and a significant degree of biochemical amplification. Thermal isomerization in a single Rho molecule at physiological temperature has been estimated to occur about once in 470 years (16). The possibility of single pheromone molecule detection by insect olfactory systems notwithstanding, the visual system is unique among sensory signal transduction systems in that it can detect single events.

The gain and kinetics of activation in the vertebrate visual transduction cascade of the rod cell are of particular interest at the interface of biochemistry and physiology. Activation of a single Rho molecule by a single photon has been estimated to prevent the entry of as many as 10^7 cations into the rod cell. Recent studies have estimated that at room temperature each $R^*$ triggers activation of cGMP PDE at rates of 1,000–2,000 molecules/s (134). During the past 5 years, transgenic animal models have been employed to study the molecular basis of rod cell physiology in vivo (139). In addition, animal models have also begun to elucidate the molecular pathophysiology of human diseases that result from defects in visual transduction or in the biosynthesis of molecules in the signal transduction pathway.

This review attempts to outline the structural basis of the molecular physiology of vertebrate Rho. It will focus on what has been learned in this respect from the recently published crystal structure of Rho (194). What insight does the structure provide about the mechanism of the “opsin shift” and spectral tuning? What is the structural basis for the incredible stability of Rho in the rod cell disk membrane in the dark? How does Rho achieve high photochemical specificity and high quantum yield? How does a single $R^*$ catalyze guanine nucleotide exchange by hundreds of $G_t$ molecules? What does the Rho structure tell us, if anything, about structure-activity relationships in other GPCRs? Finally, when possible, attempts are made to reconcile previous key findings from biochemical studies and the analysis of site-directed mutant pigments with the Rho crystal structure.

II. MOLECULAR STRUCTURE OF RHODOPSIN

A. Overview of Rhodopsin Structure

Rho is the first GPCR for which a crystal structure has been reported (194). To obtain crystals, bovine Rho was purified from rod outer segment membranes and crystallized from a detergent solution, nonyl-thiol-glucoside supplemented with the small amphiphile heptane 1,2,3-triol (186, 187). The resolution of the crystallographic data is $\sim$2.8 Å, but small segments of the cytoplasmic surface domain are not resolved. The structure represents the inactive form of Rho with its bound RET chromophore intact. A ribbon diagram of the Rho peptide...
backbone structure with the RET chromophore is presented in Figure 3. The structure discussed in this review is that of the A chain in the crystal unit cell dimer.

As an integral membrane protein, Rho comprises three topological domains: the extracellular surface, the membrane-embedded domain, and the intracellular surface. Because of the location of Rho in the disk membrane of the rod outer segment, the extracellular domain is sometimes referred to as intradiscal. The amino terminus of Rho is extracellular and the carboxy terminus is intracellular. The membrane-embedded domain consists of seven transmembrane segments (H1 to H7), which are predominantly α-helical. The helical segments form a compact bundle that contains the binding site for the RET chromophore.

B. Extracellular Surface Domain of Rhodopsin

1. Crystal structure of the extracellular surface domain

The extracellular surface domain of Rho comprises the amino-terminal tail (NT) and three interhelical loops (E1, E2, and E3) (Fig. 1). There is significant secondary structure in the extracellular domain and several intra- and interdomain interactions. The extracellular domain essentially provides a stable foundation from which the transmembrane segments extend (Fig. 3).

NT extends from the amino terminus to Pro-34 and contains five distorted strands (β1, β2, S3, S4, and S5). It is located in the crystal structure just outside of loop E3, with the side chain of Asn-2 close to that of Asp-282 in E3. The NT domain also seems to be in contact with the E3 loop in the area near Pro-12. The short segment from Gly-3 to Pro-12 forms the first two antiparallel strands in the structure (β1 and β2), which seem to lie roughly parallel to the plane of the membrane. The segment Phe-13 to Pro-34 forms strands S3 to S5, and they appear almost as a right triangle. S3 runs just outside E3 and parallel to the long axis of the molecule. S4 connects the dipeptide Ser-14/Asn-15 in NT with Pro-23, which is located close to E1. S5 (Pro-27 to Pro-34) runs along the surface of the membrane covering the extracellular space between H1 and H2. NT is glycosylated at Asn-2 and Asn-15. The oligosaccharides extend away from the extracellular domain and do not seem to interact with any part of the molecule.

In addition to the NT segment, the extracellular surface domain comprises three extracellular interhelical loops: loop E1 (amino acids 101–106) connects H2 and H3, loop E2 (amino acids 174–199) connects H4 and H5, and loop E3 (amino acids 278–285) connects H6 and H7. The E1 loop runs along the periphery of Rho. Tyr-102 interacts with Pro-23 and Glu-28 to maintain proper orientation between E1 and NT. The E2 loop is extremely interesting in that it is folded deeply into the core of the membrane-embedded region of Rho. In addition to contacts with the RET chromophore, E2 forms extensive contacts with other extracellular regions. Gly-174 and Met-183 cross the membrane surface. The Met-183 side chain points toward a hydrophobic pocket around H1, while the extended side chain of Glu-184 is surrounded by relatively hydrophilic groups and a water molecule lo-
cated close to the backbone carbonyl group of Pro-180 and the hydroxyl group of Tyr-192. The segment from Arg-177 to Glu-181 forms the β3-strand, while that from Ser-186 to Asp-190 forms the β4-strand.

The β3- and β4-strands run antiparallel. The β4-strand is situated more deeply within the membrane-embedded region of Rho than the β3-strand. The β4-strand is adjacent to the RET chromophore and forms the extracellular boundary, or roof, of the ligand-binding pocket. A disulfide bond between Cys-187 and Cys-110, which forms the extracellular end of H3, is highly conserved among all class A GPCRs. The segment from Tyr-191 to Asn-200 forms a short surface connector within E2 that is similar to both E1 and E3. The backbone carbonyl group of Tyr-191 and the amide group of the side chain of Gln-279, which is at the amino-terminal end of E3, lie in close proximity. Within the connector portion of E2, Asn-199 is near to Trp-175, although it is oriented differently in the unit cell chain B molecule.

The carboxylic acid side chain of Glu-181 in the β3 sheet of the E2 loop points toward the center of the polyene chain of the retinylidene chromophore (Fig. 4). Asp-190 at the carboxy-terminal end of the β4-strand is interesting because although it is near to the solvent-exposed surface of the receptor, its carboxy group is partially buried. Solvent access to Asp-190 may be controlled by Arg-177 and His-195, two amino acid residues with functional side chains that could act to stabilize a partially buried negative charge. Asp-190 is also strongly coupled to the chromophore by its flanking residues Ile-189 and Tyr-191 (Fig. 4).

One of the most striking and unexpected features of the Rho structure is the presence and positioning of the β4-strand (Ser-186/Cys-187/Gly-188/Ile-189), which forms the extracellular floor of the RET binding site, excluding the ligand from bulk solution on the extracellular surface. The β4-strand runs nearly parallel to the length of the polyene chain from about C9 to the Schiff base imine nitrogen. The opposite end of RET from the cyclohexenyl ring to about C10 runs along H3, which is tilted with respect to the plane of the membrane. The result is that this end of RET seems to be held very firmly in place by multiple contacts (see sect. II).

The E3 loop essentially runs along the periphery of Rho in similar fashion to E1 and to the connector segment of E2 (amino acids 191–200). E3 displays at least two potential interactions with NT. Pro-12 of NT may interact with the E3 loop, and Asn-2 is close to Asp-282.

**FIG. 4.** The RET chromophore-binding pocket of bovine Rho. A: a cut-away surface map is shown viewed from the extracellular surface. All residues above the surface of RET were removed so that the RET pocket can be appreciated. The protein is colored turquoise, and the RET is colored in red. The side chains of Trp-265 (H6) and Lys-296 (H7) are labeled for orientation. The kink in RET at the C10-C13 region of the polyene is clearly defined. B: the RET chromophore-binding pocket is shown from within the plane of the membrane bilayer. RET is colored magenta, and amino acid side chains are colored yellow except for nitrogen atoms (blue), oxygen atoms (red), and sulfur atoms (green). At least 16 amino acid residues are within 4.5 Å of the RET ligand: Glu-113, Ala-117, Thr-118, Gly-121, Glu-122, Glu-181, Ser-186, Tyr-191, Met-207, His-211, Phe-212, Phe-261, Trp-265, Tyr-268, Ala-269, and Ala-292. Some additional key amino acid residues are labeled, including the Cys-110/Cys-187 disulfide bond. RET is situated such that its proximal end (approximately C9 to C13) lies along the β4-strand and its distal end (approximately C9 to the cyclohexenyl ring) lies along H3.
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2. Structure-activity relationships in the extracellular surface domain

The role of the extracellular surface domain in Rho function has been elucidated by a number of studies involving site-directed mutagenesis. The extracellular loops and NT of bovine Rho have been shown in a deletion analysis to be important for proper folding of the receptor that allows cellular processing and chromophore binding (47). Insertional mutagenesis was also used in a related study to probe the topology of Rho and to correlate the location of epitope insertion to stability and cell trafficking (27). Interestingly, several mutations that interfered with the formation of a correct tertiary structure on the intradiscal surface resulted in mutant opsins that appeared to be retained in the endoplasmic reticulum during heterologous expression and were complexed with molecular chaperones (7). Antibody accessibility studies suggested that the NT domain constitutes a defined tertiary structure that contributes to the overall extracellular domain (35). This conclusion is supported by the crystal structure.

Rho is known to be glycosylated at Asn-2 and Asn-15 of the NT. A nonglycosylated Rho, which was prepared in the presence of tunicamycin, was defective in light-dependent activation of G\(_\text{t}\) (114). Site-directed opsin mutants with replacements of these two residues or of neighboring consensus sequence residues were studied as well. It was concluded that glycosylation at Asn-15 was required for full signal transduction activity, but apparently not for correct biosynthesis or folding (114). The structural basis of this finding is not clear from the Rho crystal structure since the oligosaccharide chains point away from the molecule and do not seem to engage in intramolecular interactions.

Future studies of mutant opsins with defects in folding, membrane insertion, or cell trafficking will be facilitated by the use of a methodology to purify regenerated pigment from nonregenerated opsin. The paradigm was developed by using the opsin mutant containing only three intradiscal Cys residues (112). It was shown that the nonregenerated opsin, which could not bind RET, was misfolded and was likely to have an incorrect disulfide bond pairing (213). This is additional evidence pointing to the role of the intradiscal domain, and in particular the early formation of a correct disulfide bond linkage between Cys-110 and Cys-187, in the proper folding of the opsin apoprotein (101).

The two conserved cysteine residues on the extracellular domain, Cys-110 and Cys-187, are essential for proper folding of opsin expressed in COS cells (112). These two residues were shown to form a disulfide linkage in an elegant study in which the four intracellular and three membrane-embedded Cys residues were removed by site-directed mutagenesis to create a mutant receptor with
only the three extracellular Cys residues remaining (111). In a related study, the double mutant C110A/C187A was shown to bind RET to form a Rho-like pigment (45). However, the meta-II-like photoproduct of the mutant pigment, which could activate Gt, in response to light, was considerably less stable than native meta-II (45). In general, mutations on the extracellular surface that might interfere with the formation of a disulfide bond between residues Cys-110 and Cys-187 correlate with a loss of function phenotype. Namely, mutations affecting the ability of these two Cys residues to juxtapose during translation or membrane translocation affect expression level, transport to the plasma membrane, and the ability of the mutant opsin to bind RET chromophore to form a pigment with normal stability. The Cys-110/Cys-187 disulfide bond seems to stabilize the ground state structure of the chromophore-binding pocket, but the adjacent side chains must be able to acquire their proper positions even without the disulfide.

Several point mutations that result in amino acid substitutions in the NT domain are linked to autosomal dominant retinitis pigmentosa (ADRP), including positions Pro-23 and Gln-28. ADRP is an inherited human disease that causes progressive retinal degeneration, loss of dim-light vision, loss of peripheral vision, and eventual blindness. Pro-23 and Gln-28 interact with Tyr-102, which is in the E1 loop. This interaction might maintain an essential structural orientation between NT and E1 that is disrupted in the NT of the ADRP mutants. Thus Tyr-102 interacts with Pro-23 and Gln-28 to maintain proper orientation between E1 and NT. The roles of specific amino acid residues in the NT domain were also studied in various transgenic mice strains harboring point mutations that correspond to sites linked to ADRP. These transgenic strains are listed in Table 1.

The amino acids of the β4-strand are highly conserved among vertebrate opsins. Of 130 opsins considered, 128 contained Ser and 2 contained Ala at position 186, 130 contained Cys at position 187, and 130 contained Gly at position 188. There was more variability at position 189, where most opsins contained an Ile, but some contain Pro or Val instead. In class A GPCRs that do not bind RET, for example, adrenergic receptors, this region does not seem to be highly conserved, except for the Cys-187 residue. Therefore, the β4-strand might serve specifically to define the RET ligand-binding pocket in vertebrate opsins.

One particularly interesting interaction in this region is that of Ile-189 with RET C19 (the methyl group bonded to C9 of the polyene chain). Because the C19-methyl group is required for light-dependent activation of Rho, it would be interesting to determine the phenotype of the mutant I189A. The prediction would be that I189A would fail to activate Gt, and would have a phenotype similar to that of 19-desmethyl Rho (opsin regenerated with the RET ana-

log that lacks the C19-methyl group) (72). Otherwise, the RET C19-methyl group might interact with another part of Rho during activation, the RET C19-methyl group-Ile-189 contact being only a ground state interaction. A mutant such as I189W might have a decreased ability to bind RET, as would any mutant with a substitution at the neighboring Gly-188 residue. If they did bind RET, the phenotypes of these mutants with respect to Gt activation would also be interesting.

In Rho, Glu-181, which arises from the linker between β3 and β4 but points toward the polyene chain, may serve a role in spectral tuning as discussed below. In addition, Glu-181 may serve to influence the electron density of the conjugated polyene system of the retinal chromophore so that photoisomerization occurs exclusively at the C11-C12 double bond. The corresponding His residue in the green and red cone pigments constitutes a part of a binding site for a chloride ion. Chloride binding causes a spectroscopic red shift in the visible absorption spectra of green and red pigments.

C. Membrane-Embedded Domain of Rhodopsin

1. Crystal structure of the membrane-embedded domain

The crystal structure of Rho suggests that 194 amino acid residues make up the seven transmembrane segments (H1 to H7) included in the membrane-embedded domain: H1 (amino acids 35–64), H2 (amino acids 71–100), H3 (amino acids 107–139), H4 (amino acids 151–173), H5 (amino acids 200–225), H6 (amino acids 247–277), and H7 (amino acids 286–306). The crystal structure of this domain is remarkable for a number of kinks and distortions of the individual transmembrane segments, which are otherwise generally α-helical in secondary structure. Many of these distortions from idealized secondary structure were not accounted for in molecular graphics models of Rho based on projection density maps obtained from cryoelectron microscopy (12, 131, 227, 249).

H1 has a very slight kink at Pro-53, its sole Pro residue. H2 begins with Pro-71, and it is also kinked around vicinal Gly residues, Gly-89 and Gly-90 so that this region of H2 is brought closer to H3 than to H1. This feature is interesting in that Gly-90 comes into close proximity to the RET Schiff base counterion, Glu-113, on H3.

H3 contains only a single Pro residue at its amino terminus, Pro-107. Consecutive Gly residues in H3 at Gly-120 and Gly-121 do not seem to distort H3 to the degree that Gly-89 and Gly-90 distort H2. The cytoplasmic end of H3 contains the highly conserved Glu/Arg/Tyr motif that is known to regulate Gt coupling. The Glu-134/Arg-135/Tyr-136 tripeptide exhibits a slight deviation from a regular helical structure. Several hydrophobic residues surround
the cytoplasmic end of H3 (Pro-71, Leu-72, Phe-148, Leu-226, Val-230, Val-250 and Met-253), which might form a binding site for Gt.

Although H4 contains two consecutive Pro residues (Pro-170 and Pro-171), significant distortion is only appreciated near the extracellular end of the segment. The cytoplasmic end of H4 is near to H2, but the two segments seem to diverge beyond the level of Trp-161, which is highly conserved in class A GPCRs. Irregular helicity is also noted in the cytoplasmic end of H4.

H5 appears to be nearly straight despite the presence of Pro-215, which is approximately in the middle of the segment. However, helix irregularity is noted, especially in the region near His-211. H5 contains six Phe residues and two Tyr residues. Tyr-223, which partially fills the interhelical region between H5 and H6, is highly conserved.

The presence of Pro-267 creates a significant bend in H6 at about the level of the Schiff base with respect to the putative membrane boundaries. The boundary between C3 and H6 at the cytoplasmic surface is highly basic due to presence of Lys-245, Lys-248, and Arg-252.

H7 is the most highly distorted of the seven transmembrane helical segments. There are kinks at two Pro residues, Pro-291 and Pro-303. In addition, the helix is irregular around the region of residue Lys-296, which is the chromophore attachment site. Pro-303 is a part of the highly conserved Asn/Pro/X/X/Tyr motif (Asn-302/Pro-303/Val-304/Ile-305/Tyr-306 in Rho).

2. Structure-activity relationships in the membrane-embedded domain

The membrane-embedded domain of Rho is characterized by the presence of several intramolecular interactions that may be important in stabilizing the ground state structure of the receptor (Fig. 5). One of the hallmarks of the molecular physiology of Rho is that it is essentially silent biochemically in the dark. The RET chromophore serves as a potent pharmacological inverse agonist to minimize activity. The result is that the rod cell can attain single photon sensitivity (94). The Rho structure reveals numerous potentially stabilizing intramolecular interactions, some mediated by the RET chromophore and some arising mainly from interhelical interactions that do not involve the RET-binding pocket directly.

The phenyl rings of the side chains of Phe-293 and Phe-294, both in H7, interact with Leu-40 in H1 and Cys-264 in H6, respectively. This interaction seems to be facilitated by the slight distortion of H6 in the region near Ile-263. In addition to these core interactions, there appear to be four H-bond networks that provide stabilizing interhelical interactions at or near the cytoplasmic surface of the receptor.

H-bond network 1 links H1, H2, and H7. The helical structure of H7 is elongated in the region from Ala-295 to Tyr-301, which permits the backbone carbonyl group of Ala-299 to H-bond with the side chains of Asn-55 in H1 and Asp-83 in H2. Asn-55 is a very highly conserved residue that plays the central role in the H-bond network 1 since it H-bonds to both Asp-83 and the backbone carbonyl of
Ala-299. Asp-83, in turn, is connected to the backbone carbonyl of Gly-120 in H3 through a water molecule.

H-bond network 2 links H2, H3, and H4. This network involves Asn-78 as the key residue, which H-bonds to the hydroxyl functions of Ser-127 (H3), Thr-160 (H4), Trp-161 (H4), and the backbone carbonyl of Phe-159. Mutant pigments S127A and T160V displayed normal ground-state spectral properties consistent with a lack of direct contact with RET (103). Another possible interhelical interaction in this region might involve Glu-122 (H3), Met-163 (H4), and His-211 (H5). An indirect functional interaction between Glu-122 and His-211 has been demonstrated experimentally (18).

H-bond network 3 links H3 and H6. This network involves the conserved Arg-135, which interacts with Glu-134 and with the hydroxyl group of Thr-251 and side chain of Glu-247. The carboxylate of Glu-134 seems to be in position to form a salt bridge with the guanidinium group of Arg-135. This would be consistent with the hypothesis that Glu-134 is unprotonated in Rho and becomes protonated during the transition to R* (9, 59). It is interesting to note the three consecutive Val residues (Val-137, Val-138, and Val-139) are situated to form a cytoplasmic cap to H3 so that the Ghu-134/Arg-135 dipptide is between the receptor core and the Val tripeptide. This Val cap might act to stabilize the Glu-134/Arg-135 salt bridge, which in turn acts to keep the receptor in its off state in the dark. It is also interesting to note that Thr-251 in Rho is in the position equivalent to Ala-293 in the α1B-adrenergic receptor. Mutation of Ala-293 causes the receptor to become constitutively active (121). The Asp(Glu)/Arg/Tyr(Trp) motif at the cytoplasmic border of H3 is one of the most highly conserved structural motifs in class A GPCRs.

Finally, H-bond network 4 links H6 and H7. The key interaction here is between Met-257 and Asn-302. The precise functional importance of the highly conserved Asn/Pro/X/X/Tyr motif (Asn-302/Pro-303/Val-304/Ile-305/Tyr-306 in Rho) is unclear. However, one key structural role is to mediate several interhelical interactions. The side chains of Asn-302 and Tyr-306 project toward the center of the helical bundle. The hydroxyl group of Tyr-306 is close to Asn-73 (cytoplasmic border of H2), which is also highly conserved. A key structural water molecule may facilitate an H-bond interaction between Asn-302 and Asp-83 (H2). A recent mutagenesis study of the human platelet-activating factor receptor showed that replacement of amino acids at the positions equivalent to Asp-78 and Asn-302 in Rho with residues that could not H-bond prevented agonist-dependent receptor internalization and G protein activation (137).

The interaction between Met-257 and the Asn/Pro/X/X/Tyr motif was predicted earlier to explain the results of a mutagenesis study in which Met-257 was replaced by each of 19 other amino acid residues (91). Nearly all Met-257 replacements caused constitutive activity of the mutant opsins. A decrease in interaction between Met-257 and Asn-302 might relieve an interhelical constraint that stabilizes the ground state structure of Rho. However, the most highly constitutively active Met-257 mutants were M257Y, M257N, and M257S, which are all theoretically capable of forming H-bonds with the adjacent Asn-302. It is conceivable that the amino acid residue at position 257 in a mutant receptor forms H-bond interactions that stabilize the active state structure of the receptor as well. Whether constitutive activity is caused simply by a lack of H6/H7 interactions, or whether a gain of active state stabilizing interactions is required could be determined by testing mutant receptors with alterations of the Asn/Pro/X/X/Tyr motif, for example, N302A and I305A in Rho, or analogous mutations in other class A GPCRs.

It should be noted that the highly conserved Asn/Pro/X/X/Tyr motif and Glu/Arg/Tyr (H3) motifs play central roles in several of the core-stabilizing interactions described above. Their precise roles in the regulation of receptor activation will require knowledge about any structural reorientations that occur with respect to these residues in the formation of R*.

D. Chromophore-Binding Pocket

1. Crystal structure of the chromophore-binding pocket

The RET chromophore is a derivative of vitamin A1 with a total of 20 carbon atoms (Fig. 1). The carbon atoms of the cyclohexenyl ring are numbered C1 to C6. The polyene carbons extend from C7 to C15. Two methyl groups (C16 and C17) are bonded to C1, and single methyl groups are attached at each of three other carbons: C5 (C18 methyl), C9 (C19 methyl), and C13 (C20 methyl). The structural conformation of the bound chromophore in the Rho crystal structure appears to be 6-s-cis, 11-cis, 12-s-trans. The protonated Schiff base bond appears to be in the anti conformation. A higher resolution Rho structure would be required for a crystallographic determination of the precise chromophore structure. Although a variety of spectroscopic studies support the 6-s-cis, 11-cis, 12-s-trans RET conformation, recent NMR experiments suggested a 6-s-trans conformation (80).

The binding site of the RET chromophore lies within the membrane-embedded domain of the receptor (Fig. 4). All seven transmembrane segments and part of the extracellular domain contribute interactions with the bound chromophore. The chromophore is located closer to the extracellular side of the transmembrane domain of the receptor than to the cytoplasmic side. The chromophore polype from C5 to C11 runs almost parallel to H3, which provides many of the amino acid side chains that form the chromophore-binding pocket: Glu-113, Gly-114, Ala-117, Thr-118, Gly-120, and Gly-121. The polypeptide chain facing toward the
extracellular side of the receptor is covered, or capped, by the amino acid residues from the β4-sheet (Ser-186 to Ile-189) of the E2 loop as described above. The carboxylic acid side chain of Glu-181 in the β3-sheet of the E2 loop points toward the center of the RET polypeptide chain.

Glu-113 serves as the RET Schiff base counterion. A number of other amino acid side chains surround the imine moiety, including Tyr-43, Met-44, and Leu-47 in H1, Thr-94 in H2, and Phe-293 in H7. In particular, Met-44 and Leu-47, in addition to the peptide bond between Phe-293 and Phe-294, help to orient the side chain of Lys-296 in the direction of the long axis of Rho. The phenyl rings of Phe-293 and Phe-294 also interact with side chains of adjacent helices. The two oxygen atoms of the Glu-113 carboxylate side chain of Glu-113 are located 3.3 and 3.5 Å from the imine nitrogen. The hydroxyl group of Thr-94 is also ~3.4 Å from one of the Glu-113 carboxylate oxygens. Thr-92 and Thr-93 are also in the vicinity of the Schiff base imine but may not be close enough to contribute significantly to stabilization of its protonated ground state. The presence of water molecules in the Schiff base region has been postulated, but the crystal structure at the reported resolution does not contain defined water in this region (168).

The position of the cyclohexenyl ring of the chromophore is largely constrained on the cytoplasmic side of the binding pocket by three residues: Glu-122 (H3), Phe-261 (H6), and Trp-265 (H6). The indole side chain of Trp-265 points inward from the more cytoplasmic position of the Trp-265 backbone and comes within ~3.8 Å of the RET C=C double bond and C=C orientation (Phe-261 Cα-Gly-121 Cα distance 3.5 Å). Gly-121 interacts with RET in a direct steric manner and lies closest to the C=C double bond of the cyclohexenyl ring (Gly-121 Cα-RET C=C double bond distance 3.5 Å). Gly-121 is close to Phe-261 (H6) in the Rho crystal structure. They pair to form one boundary of the RET binding site, defining the C4=C5=C18 orientation (Phe-261 Cα-Gly-121 Cα distance 5 Å; Phe-261 Cα-RET C4=C5=C18 orientation 3.7 Å). This portion of the RET binding pocket around H3 and H6 appears to be held rigidly together by tight van der Waals interactions.

H4 contributes only one residue, Cys-167, directly to the chromophore-binding pocket.

In summary, at least 16 amino acid residues that define the structure and function of the retinal chromophore in Rho. The Schiff base linkage of the chromophore to Lys-296 is a key feature of Rho structure (92). Light-dependent Schiff base deprotonation is required for the formation of the active state of the receptor R* (117, 153). However, it has been shown that light can induce the receptor active state in the absence of a Schiff base chromophore linkage to the opsin (268). In this experiment, mutant opsin K296G was treated with a model retinal Schiff base compound prepared from 11-cis-retinal and n-butyl amine. A visible pigment was formed, although no linkage was present between the model compound and the opsin. Photolysis of the model chromophore led to receptor activation as judged by Gt activation. This demonstrates that opsin can employ a diffusible ligand, which is analogous to ligand-dependent GPCRs, such as biogenic amine receptors (189).

Glu-113 in bovine Rho serves as the counterion to the positive charge of the RET protonated Schiff base (171, 225, 267). Glu-113 is unprotonated and negatively charged in the ground state of Rho (57). It becomes protonated upon light-dependent formation of meta-II and is the net proton acceptor for the Schiff base proton (104). The Glu-113-protonated Schiff base interaction serves to stabilize the Schiff base proton such that its acid dissociation constant (pKₐ) in Rho is estimated to be ~12, compared with a value of ~7 for a model compound in aqueous solution, although the mechanism of protonated Schiff base stabilization is not entirely clear from the crystal structure. The stable interaction between Glu-113 and the protonated Schiff base may also inhibit hydrolysis of the Schiff base linkage in darkness. For example, hydroxylamine does not react with the Schiff base of Rho, but readily reacts with that of meta-II or with Rho mutants in which Glu-113 is replaced by a neutral amino acid residue by mutagenesis (225).

This is an important consideration since the opsin
alone, without the RET chromophore, has a small but measurable activity (31, 207, 215, 244). The high sensitivity of the rod cells depends on an extremely low intrinsic level of signaling in darkness. Dark noise can be generated by thermal isomerization events in Rho (14, 15, 262), by the presence of opsin lacking the RET chromophore, which acts as an inverse agonist, or by mutant opsins that display the property of constitutive activity (215). Constitutive activity refers to the ability of an opsin to activate Gt in the absence of any chromophore. Generally, a mutation that disrupts a putative salt bridge between Glu-113 and Lys-296 in the opsin apoprotein leads to constitutive activity. Replacement of either Glu-113 or Lys-296 by a neutral amino acid results in a mutant opsin with constitutive activity.

Other mutations such as G90D or A292E also result in constitutive activity, presumably because the introduction of the negatively charged residue into the membrane-embedded domain of the receptor affects the stability of the Glu-113/Lys-296 salt bridge (44, 207). The crystal structure shows that H2 is kinked around vicinal Gly residues, Gly-89 and Gly-90, so that this region of H2 is brought closer to H3 than to H1. This feature is interesting in that Gly-90 comes into close proximity to the retinylidene Schiff base counterion, Glu-113, on H3. A mutation that results in the replacement of Gly-90 by an Asp residue causes congenital stationary night blindness in humans, probably because of destabilization of the ionic interaction between Glu-113 and the Schiff base (269), or because of constitutive activity of the mutant opsin apoprotein that results from a disruption of a salt bridge between Glu-113 and Lys-296 (207). The mechanism of constitutive activity of opsins and the potential relevance of constitutive activity to visual diseases such as congenital night blindness has been reviewed (208).

Osin activity was reported to be only $\sim 10^{-6}$ as much as the activity of meta-II, a level much lower than previously estimated (159). Therefore, the role of opsin activity in bleaching desensitization and the pathophysiology of retinal degeneration may be much less than previously suggested. Opsin can also be activated without light by the addition of all-trans-retinal or various retinal analogs (31, 220). Expressed mutant opsins can also be activated by various retinal analogs (88). These results show that diffusible ligands can activate opsin and provide information about the active-state conformation of Rho.

Glu-113 is not located near C12 of the retinal polyene as predicted by two-photon spectroscopy and NMR spectroscopy of retinal analogs and semiempirical quantum mechanical orbital calculations (24, 82, 80, 90). However, the C12 of RET does interact with Glu-181 from the E2 loop. Glu-181 may serve to influence the electron density of the conjugated polyene system of the RET chromophore so that photoisomerization occurs exclusively at the C11-C12 double bond. The potential ionic interaction between RET and Glu-181 also suggests that it may have a role in the mechanism of the opsin shift (see below). Perturbation of the electron distribution near the center of the polyene chain is one mechanism to facilitate spectral tuning (97).

Glu-181 is highly conserved among vertebrate opsins, blue and ultraviolet (UV) cone pigments. The corresponding position in green and red cone pigments is His-197, which forms part of a chloride ion-binding site. Chloride binding causes a red shift in absorption of the green and red pigments. Interestingly, the H197E/R200Q mutant of the human green cone pigment displays a visible $\lambda_{\text{max}}$ value of 500 nm (257), which is the same as the $\lambda_{\text{max}}$ value of Rho, suggesting that perturbation of the polyene by chloride may be the only element in the green cone pigment responsible for its spectral difference from Rho. The negative charge of the chloride ion bound to His-197 in the long-wavelength sensing cone pigments might be brought closer to RET than the charge of the carboxylate of Glu-181 in Rho.

The position of the cyclohexenyl ring of the chromophore is largely constrained on the cytoplasmic side of the binding pocket by three residues: Glu-122 (H3), Phe-261 (H6), and Trp-265 (H6). The indole side chain of Trp-265 points inward from the more cytoplasmic position of the Trp-265 backbone and comes within $\sim 3.8 \AA$ of the RET C20. Trp-265 is close enough to RET that it can serve as an intrinsic probe of the chromophore conformation (149). Side chains from Met-207, His-211, and Phe-212 on H5 and Tyr-268 and Ala-269 on H6 further constrain the chromophore ring. Replacements of Phe-261 by Tyr or Ala-269 by Thr produce bathochromic spectral shifts in the $\lambda_{\text{max}}$ values of the resulting mutant pigments (38). These residues are also responsible in part for the spectral shift in red cone pigments. Whereas red pigments have Thr and Tyr at the positions corresponding to 261 and 269 in Rho, green cone pigments have Phe and Ala (11, 181).

The interaction between Gly-121 and RET is consistent with mutagenesis experiments in which replacement of Gly-121 caused blue-shifted $\lambda_{\text{max}}$ values and decreased RET binding that corresponded to the bulk of the substituted side chain (86). Second-site replacement of Phe-261 by Ala caused a reversion of the loss of function Gly-121 mutant phenotypes, which was interpreted to mean that Gly-121 and Phe-261 interacted to form a part of the RET binding pocket (85). Gly-121 and Phe-261 are indeed very close together in the Rho crystal structure. They pair to form one boundary of the RET binding site to define the C4-C5-C18 orientation (Phe-261 Cz-Gly-121 Cz distance 5 Å; Phe-261 Cz-RET Cz distance 3.7 Å). Interestingly, Gly-121 is conserved among all vertebrate and invertebrate visual pigments (204), and Phe-261 is strictly conserved among nearly all GPCRs (3). As described above, in long-wave-
length sensing cone pigments, Phe-261 is replaced by a tyrosine that is involved in spectral tuning (38).

The Rho structure shows no interaction between Gly-121 and RET C_{19} as was suggested by data from regeneration of mutants with various chromophore analogs (S3, S4). However, the structure does not rule out the possibility that the two are coupled together during activation, or that they may come into proximity in R^*, especially considering that Gly-121 replacement mutants exhibit dark activity even with bound RET (S3, S7). In these mutant opsins, RET displays partial agonist activity. Regeneration of opsins with C_{20}-desmethyl retinal also produces a pigment with an increased level of basal activity in the dark.

### E. Cytoplasmic Surface Domain of Rhodopsin

1. Crystal structure of the cytoplasmic surface domain

The cytoplasmic domain of Rho comprises three cytoplasmic loops and the carboxy-terminal tail: C1 (amino acids 65–70), C2 (amino acids 140–150), C3 (amino acids 226–246), and CT (amino acids 307–348). Loops C1 and C2 are resolved in the crystal structure, but only residues 226 to 235 and 240 to 246 are resolved in C3. CT is divided into two structural domains. C4 extends from the cytoplasmic end of H7 at Ile-307 to Gly-324, just beyond two vicinal Cys residues (Cys-322 and Cys-323), which are posttranslationally palmitoylated. The remainder of CT extends from Lys-325 to the carboxy terminus of Rho at Ala-348. The crystal structure does not resolve residues 328 to 333 in CT.

Loop C1 displays a rigid structural organization that includes the basic side chains of His-65, Lys-66, Lys-67, and Arg-69. The Lys-66 and Arg-69 side chains point toward the bilayer. His-65 sits close to the C4 loop at its H8 border. Lys-67 points toward the solvent and seems to interact with CT, which runs nearly parallel to C1.

Loop C2 resembles an L-shaped structure when it is viewed from a vantage point parallel to the putative membrane plane. A short β-barrel structure extends from Met-143 to Phe-146 along the main long axis of Rho. A distinct cytoplasmic border seems to be formed by the four polar side chains of Lys-141, Ser-144, Asn-145, and Arg-147. C2 and C3 extend approximately to the same level above what appears to be the cytoplasmic border of the receptor.

H5 probably extends into the cytoplasmic region but breaks at the start of C3 at Leu-226, which is immediately followed by an S-shaped loop structure that lies nearly on the membrane surface. The loop extends nearly to the lipid-facing side of H6 at Ala-235 and leaves a part of the cytoplasmic surface of the seven helical bundle uncovered. The region from Gln-236 to Glu-239 is absent in the crystal structure. The polar side chains of Ser-240 and Thr-242 come close to a portion of CT around Ser-334 to form a small cluster of hydroxyl groups. The tetrapeptide Thr-243 to Ala-246 is α-helical.

The hallmark of the C4 loop is an α-helical stretch, H8. H8 is connected to H7 by the Met-309/Asn-310/Lys-311 tripeptide that acts as a short linker. H8 lies nearly perpendicular to H7 and together with the Asn/Pro/X/Tyr motif in H7 is one of the most highly conserved long stretches of primary structure in Rho. The environment around H8 is mainly hydrophobic, which may lead to increased helical stability. H8 might be best described as a cationic amphipathic α-helix, with Lys-311 and Arg-314 on one face of the helix and Phe-313, Met-317, and Leu-321 buried in the hydrophobic core of the bilayer between H1 and H7. H8 points away from the center of Rho, and it appears that the palmitoyl groups linked to Cys-322 and Cys-323 by thioester bonds may be anchored in the membrane bilayer, although this is not resolved in the crystal structure. The helical structure of H8 is terminated by Gly-324.

The residues at the extreme carboxy-terminal end of CT compose the most solvent exposed region of Rho. CT folds back over a small portion of the helical bundle at H1 and H7.

2. Structure-activity relationships in the cytoplasmic domain of rhodopsin

A number of cytoplasmic proteins are known to interact exclusively with R^*. Because the crystal structure depicts the inactive Rho structure that does not interact significantly with cytoplasmic proteins, the structure can provide only indirect information about the relevant R^* state. In addition, two regions of the cytoplasmic surface domain of Rho (amino acid residues 236–239 and 328–333) are not fully resolved in the crystal structure. Potentially important structural information relevant to understanding protein-protein interactions in the visual transduction cascade may be lacking in the reported structure. It should also be noted that the borders between the transmembrane helical segments and the cytoplasmic loops do not necessarily represent the boundary between aqueous phase and membrane bilayer. The helical segments generally tend to extend into the cytoplasmic aqueous phase. A number of amino acid residues that are involved in G_i binding or activation, such as Glu-134 or Lys-248, are situated in the helical segments.

Perhaps the most extensively studied receptor-G protein interaction is that of bovine Rho with G_i (96). Detailed biochemical and biophysical analysis of the R^*-G_i interaction has been aided by mutagenesis of the cytoplasmic domain of bovine Rho. Numerous Rho mutants defective in the ability to activate G_i have been identified (69). Several of these mutant receptors were studied by flash photolysis (55, 68), light scattering (54), or proton
uptake assays (9). The key overall result of these studies is that C2, C3, and H8 are involved in R*–Gt interaction. This finding is consistent with other approaches including peptide competition studies (129, 155).

Although several biochemical studies implicated CT in Gt activation (201, 247), alanine-scanning mutagenesis failed to confirm that the proximal portion of CT was required (191, 259). Recently, a combination of site-directed mutagenesis and peptide binding studies clearly showed that the C4 loop region, which includes H8, is involved in Gt binding and activation (55, 120, 155). Direct evidence for the interaction between H8 and Gt comes from studies using a synthetic peptide corresponding to Asn-310 to Leu-321 of Rho, which was shown to bind to Gt (155).

H8 is a cationic amphipathic helix that may bind a phospholipid molecule, especially a negatively charged phospholipid such as phosphatidylserine. In fact, spectroscopic evidence has been reported to show an interaction between Rho and a lipid molecule that is altered in the transition of Rho to meta-II (19, 103). High conservation of Phe-313 and Arg-314 suggests that the amphipathic character of H8 may be functionally important. H8 points away from the center of Rho, and the area of the membrane surface covered by the entire cytoplasmic surface domain appears to be roughly large enough to accommodate Gt in a one-to-one complex.

The CT distal to the palmitoylated Cys-322 and Cys-323 residues appears to be highly disordered and dynamic based on the results of site-directed electron paramagnetic resonance (EPR) spin labeling (135). However, the CT appears to encode essential information concerning Rho sorting and targeting. Mutation of Pro-347 is associated with a biosynthetic cellular transport defect that leads to ADRP (243). In addition, CT interacts with a cytoplasmic dynein light-chain Tctex-1. The Rho-dynein interaction controls the polarized targeting of Rho in the rod cell and in polarized epithelial cells when ectopically expressed (246).

Conformational changes in the cytoplasmic surface domain that are coupled to chromophore isomerization are discussed in detail in section IV.

III. MECHANISM OF THE OPSIN SHIFT AND SPECTRAL TUNING IN VISUAL PIGMENTS

The molecular interactions between the opsin protein and RET define the spectral properties of a particular visual pigment. The broad visible absorption peaks of visual pigments represent a collection of overlapping energy states. The absorbance at a particular wavelength can be viewed as the energy difference between the chromophore ground state energy and the energy of its first excited state. An increase in the energy gap causes a blue shift (hypsochromic shift) from a particular reference value. A decrease in the energy gap causes a red shift (bathochromic shift) from a particular reference value.

Rho has been used as a model pigment for a variety of chemical and spectroscopic studies to elucidate the mechanism of the opsin shift. In this context, the opsin shift may be defined as the difference between the $\lambda_{\text{max}}$ value of a RET Schiff base model compound (~440 nm) in solution and that of Rho (500 nm). How is the positive charge on the protonated Schiff base, which can be delocalized by the conjugated polyene system, stabilized by the protein to create a unique envelope of chromophore electronic distribution? One important result to arise from the study of mutant bovine Rho pigments was the identification of Glu-113 as the RET Schiff base counterion (171, 225, 267). This result combined with the characterization of other membrane-embedded carboxylic acid residues by both UV-visible spectroscopy (169, 171, 225, 267) and microprobe resonance Raman spectroscopy (151) suggested that the retinal-binding pocket in bovine Rho was electrostatically neutral (24). Additional studies including the use of photoaffinity reagents (170, 266), retinal analogs regenerated with site-directed mutants (212), or site-directed mutant pigments (85, 86, 149, 169) led to a more complete picture of the amino acid residues in the membrane-embedded domain of Rho that interact with the retinal chromophore.

The crystal structure now provides a clear picture of the RET chromophore binding pocket in Rho (Fig. 4). One of the key surprises with respect to previous models of the opsin shift mechanism is Glu-181, which lies in the extracellular linker between $\beta_3$ and $\beta_4$ and points toward the center of the polyene chain. Glu-181 is highly conserved among vertebrate opsins, and blue and UV cone pigments. However, the corresponding position in green and red cone pigments is His-197. An extensive mutagenesis study of positively charged amino acids in human middle (M-) and long (L-) wavelength sensitive pigments identified His-197 and Lys-200 in E2 as the chloride ion binding site in these pigments (257). Chloride binding in the M- and L-sensing pigments causes a significant spectral red shift. It seems plausible that a chloride ion held in place by His-197 and Lys-200 might interact with the central portion of the polyene of RET in these pigments.

Although the role of Glu-181 in the opsin shift in Rho has not yet been elucidated, data available to date suggest that the dominant mechanism responsible for the opsin shift is the interaction of dipolar amino acid residues with both the ground-state and excited-state charge distributions of the chromophore (127, 150). This general mechanism is supported by the crystal structure in that the RET binding pocket contains a large number of dipolar or polarizable amino acid residues.

Because the visual pigments of many species have been studied by absorption spectroscopy or microspec-
trophotometry of visual organs, it is straightforward to classify visual pigments solely on their visible $\lambda_{\text{max}}$ values. Another common classification system is to group visual pigments based on the photoreceptor cell type of the retina in which they are found (29). Rod cells, responsible for dim-light (scotopic) vision, contain Rho (“red” opsin). Cone cells, responsible for bright-light (photopic) and color vision, contain iodopsins (“violet” opsins), also known as cone pigments or color vision pigments. The cloning and characterization of opsins from a variety of species have allowed further comparisons and phylogenetic classifications based on their structural, spectral, and biochemical properties (8). The homology in the opsin family of genes indicates that divergent evolution occurred from a single precursor RET-binding protein to form short (S-) and L-wavelength absorbing ancestors. The L-sensing ancestor diverged to form L- and M-sensing pigments. The S-wavelength sensing ancestor then diverged to form an S-sensing pigment and the family of Rhos and Rho-like M-sensing pigments. These evolutionary models can be tested experimentally by ancestral gene reconstruction using gene synthesis methodology (39, 40).

Whereas all Rho pigments generally absorb maximally at ~500 nm, the $\lambda_{\text{max}}$ values of cone cell pigments vary. Individual cone pigments display $\lambda_{\text{max}}$ values from the near UV (~380 nm) to the far visible red (~600 nm). Different vertebrate species may have more or fewer cone types, but all cone pigments share the same RET (or 11-cis-3,4-didehydroretinylidene) chromophore. Therefore, any differences in spectral properties among a collection of cone pigments must be related to differences in the structures of their RET binding pockets, which is ultimately related to amino acid sequence, although not necessarily strictly related. Cone pigments also display a number of other distinct biochemical properties, including fast rates of R* decay and chromophore regeneration compared with those of Rho. Specific amino acid residues in the chromophore-binding pocket seem to control these biochemical properties independently of spectral tuning (102).

Despite two centuries of study, the genetics, physiology, and psychophysics of human color vision remain of great interest. Human trichromatic color vision requires the presence of three physiological detection systems, which correspond to the three classes of photoreceptor cone cells. Each cone class (S-wavelength sensing, M-wavelength sensing, and L-wavelength sensing) contains a different pigment molecule. The peaks of absorbance are different for the three cone-cell pigments, but their absorbance spectra overlap considerably so that a combination of one, two, or three cone types reacts more or less strongly to a given light stimulus. A key element is that the response of a particular cone cell is the same no matter what the energy of the photon that it captures (i.e., as long as chromophore isomerization occurs). Only the efficiency of photon capture varies with photon energy and the dynamic output relates only to the rate of photon capture. For example, in the simplest case of a human retina with only three individual cone cells, one cell of each class, chromatic lights would be perceived to be identical if they produced the same absorptions in all three cells, and different if they did not. Thus human trichromatic vision is the result of three independent comparisons of rates of photon absorption by the three cone types.

Three genomic and cDNA clones encoding the apo-proteins of the human cone pigments were cloned and characterized (177). The amino acid sequences of these opsins are ~41% identical to that of human Rho. The M- and L-sensing opsins are ~90% identical to each other and ~43% identical to the S-sensing opsin. Previously, the spectral properties of human cone pigments had been studied by a variety of techniques ranging from psychophysical color matching to microspectrophotometry (29). More recently, the human cone pigment genes were expressed in tissue culture cell lines, reconstituted with RET, and studied by UV-visible spectroscopy (160, 190). The $\lambda_{\text{max}}$ values reported in two studies were as follows: S 426 nm, M 530 nm, L 552 nm and 557 nm for polymorphic variants (160); and S 424 nm, M 530 nm, and L 560 nm (190). The $\lambda_{\text{max}}$ value of the human S-sensing pigment was recently reported to be 414 nm from a measurement of its absolute spectrum (66). These studies also confirmed the correct functional assignments of the pigments that had been based on genetic analysis of the cloned pigment genes.

Analysis of the arrangement of the cone opsin genes on the X chromosome has led to a preliminary understanding of the molecular genetics of inherited variations in color vision (176). In males with normal color vision it was proposed that a single L-sensing opsin gene resides with one or more M-sensing opsin genes in a head-to-tail tandem array. Thus, in one type of color vision defect, anomalous trichromacy, unequal intragenic recombination can theoretically result in an opsin gene that is a hybrid between M- and L-sensing genes. It was proposed that these hybrids would have anomalous spectral properties (176, 179). This hypothesis was confirmed experimentally at the level of the photoreceptor pigment by obtaining absorption spectra for heterologously expressed hybrid pigments responsible for anomalous trichromacy (161). Quantitative polymerase chain reaction (PCR) methods have been applied to evaluate in more detail the numbers and ratios of M- and L-wavelength sensing cone opsin genes in males with normal color vision. It was found that many subjects had more numerous cone opsin genes than previously suggested and that in many cases more than one L-wavelength opsin gene was present on the X chromosome (178, 180).
molecular genetics of blue cone monochromacy have also been investigated (173). A genetic model to account for the absence of the green and red genes has been tested in transgenic mice (256). The results suggest that a conserved 5'-region interacts with the green or red gene promoter to determine which gene is expressed in a given cone cell.

IV. MOLECULAR MECHANISM OF RECEPTOR ACTIVATION

Although the crystal structure of Rho does not provide direct information about the structure of R* or about the dynamics of the Rho to R* transition, it does provide a wealth of information that should help to design experiments using existing methods to address specific questions regarding the molecular mechanism of Rho activation. An inactive receptor conformation must be capable of changing to an active conformation, which catalyzes nucleotide exchange by a G protein. In Rho, the RET chromophore is in its "off" state, but switches to the "on" state 11-trans geometry by photoisomerization leading to the R* conformation of the receptor. Any amino acid side chain that is involved in such a conformational change must be able to exist in two different sterical and/or electrostatic states, which can be designated as on or off depending on whether the particular state is observed in the active or inactive receptor, respectively (62, 245).

Because receptor activation involves conformational changes at different topological locations, an individual amino acid or a structural domain in the chromophore-binding pocket may be in its on state without necessarily locking the G interaction domain into its active conformation, and vice versa. Thus a concerted transition of individual groups may create the overall active conformation, but it may not be clear whether or not all of the measurable structural changes are actually essential. A minimal subset of molecular groups may be able to govern the transition to an active receptor conformation irrespective of the binary states (off/on) of all possible groups usually participating in receptor activation. The functional hierarchy of individual group transitions may become obvious only in mutant, or in otherwise modified receptors, in which the state of an individual amino acid side chain can be influenced, or even locked, into one of two states. Ideally, it should be possible to alter the receptor activation pathway such that certain transitions become uncoupled from other transitions. It has been argued that the simplified binary model of individual molecular transitions provides a framework to analyze functional data obtained from recombinant Rhos (62, 91).

According to this argument, only molecular changes occurring between dark Rho and the R* state are relevant. Thus FTIR-difference spectroscopy has proven to be a well-suited technique for the study of light-induced conformational changes in retinal proteins (60, 218, 229). FTIR-difference spectroscopy measures frequency shifts of only those vibrational modes that are affected during photoproduction formation, irrespective of whether they are caused by the protein constituents or by the chromophore. In contrast, resonance Raman spectroscopy measures specifically chromophore vibrations and thereby helps to define the chromophore geometry and the Schiff base protonation state without the potential drawback of overlapping absorptions of amino acid side chains. Both techniques have contributed significantly to knowledge of molecular changes occurring during meta-II formation. Application of these techniques to site-directed Rho mutants has allowed the identification of a number of on/off states of particular amino acids during R* formation.

For example, among the membrane-embedded carboxylic acid groups, light-induced changes of protonation states or hydrogen-bond strengths were deduced from characteristic frequency shifts of C=O stretching vibrations of protonated carboxylic acid groups in FTIR-difference spectra. Their assignment to specific Asp or Glu residues was based on the disappearance of specific difference bands in site-directed mutants and revealed that Asp-83 (57, 209) and Glu-122 (57) are protonated in both dark Rho and meta-II, whereas Glu-113 is ionized in the dark state and becomes protonated in meta-II (104).

Evidence for the importance of steric interactions distal to the Schiff base comes from FTIR studies using ring-modified retinal analogs. Increased flexibility, as in 5,6-dihydro (71) or 7,8-dihydro analogs (196), reduces the usually observed torsions along the retinal chain in the intermediate trapped at 80 K where bathorhodopsin would normally be stable. In addition, the protein conformational changes observed at temperatures that stabilize the meta-I or meta-II intermediates differ from those observed in native Rho. In an extreme case, illumination of a pigment regenerated with a retinal analog lacking the cyclohexenyl ring fails to induce the complete set of infrared absorption changes typical of the meta-II conformation and results in reduced G0 activation (105). Therefore, the cyclohexenyl ring must transmit important steric changes to the protein. This model seems consistent with the location of the RET ring in the crystal structure of Rho.

Movement of α-helical domains is known to be involved in the signal transduction mechanisms of some TM receptor proteins, such as the bacterial chemoreceptors (162), and has been shown to occur during the proton pumping cycle following retinal isomerization in bacteriorhodopsin (BR), the seven-transmembrane segment light-driven proton pump (237–239). Recent studies have suggested that steric and/or electrostatic changes in the ligand-binding pocket of Rho may cause changes in the
relative disposition of TM helices within the core of the receptor. These changes may be responsible for transmitting a “signal” from the membrane-embedded binding site to the cytoplasmic surface of the receptor. Trp mutagenesis (149), mutagenesis of conserved amino acid residues on H3 and H6 (85, 86), and the introduction of pairs of His residues at the cytoplasmic borders of TM helices to create sites for metal chelation (228) have recently provided insights regarding the functional role of specific helix-helix interactions in Rho.

The indole group of a Trp amino acid residue is often used as a noninvasive environment-sensitive probe of protein structure because of its unique absorption and fluorescence properties. UV-absorption spectroscopy has suggested that the local protein environment around Trp residues changes during the conversion of Rho to meta-II (205). In addition, a linear dichroism study of UV-difference bands indicated a reorientation of an indole side chain during the meta-I to meta-II conversion (37). To determine which Trp residues were responsible for the absorbance changes previously reported, each of the five Trp residues in Rho was replaced by either a phenylalanine or a tyrosine. Replacements of Trp-126 and Trp-265 resulted in a decrease in the magnitudes of the UV-difference peaks at 294 and 302 nm. These results were consistent with a change of Trp-126 and Trp-265 to more polar environments during activation of the receptor (149). It was further suggested that the photoactivation of Rho involved a change in the relative disposition of H3 and H6, which contain Trp-126 and Trp-265, respectively, within the α-helical bundle of the receptor.

H3 of Rho contributes many amino acid side chains that interact with RET, including Glu-113 and Gly-121. Replacement of Gly-121 resulted in a relative change of opsin selectivity for reconstitution with all-trans-retinal over RET in membranes. All-trans-retinal could efficiently bind and activate the mutant opsins, even though none of them displayed constitutive activity. The G121L replacement also caused RET to become a partial agonist in the G121L pigment (87). These results showed that H3, and Gly-121 in particular, were important in determining the specificity of the chromophore-binding pocket in Rho. The cyclohexenyl ring portion of the RET binding site is bordered by Gly-121 and Phe-261, consistent with the notion that functional interactions between H3 and H6 mediated by the chromophore are crucial for receptor photoactivation. Phe-261 was also found to be involved in the control of the decay of an early photointermediate (blue shifted intermediate) in a study of site-directed mutants by laser photolysis and rapid time-resolved absorption difference spectroscopy (106). Similar measurements of recombinant expressed Rho and Glu-113 counterion mutants were also recorded to provide a basis for understanding the relative movement of H3 and H6 required for receptor activation (107).

The functional interaction of H3 and H6 was further probed in a study in which metal ion binding sites were introduced between the cytoplasmic surfaces of transmembrane helices with the aim of restraining specific activation-induced conformational changes (228). Pairs of His residues are capable of chelating metal ions such as Zn(II) if the distance and geometry between the residues is appropriate. His residues substituted for the native amino acids at the cytoplasmic ends of H3 and H6, but not H5 and H7, created mutant proteins that were able to activate Gt in the absence, but not in the presence, of metal ions. It was concluded that specific metal-ion cross-links between positions 138 and 251, or 141 and 251, on H3 and H6, respectively, prevented receptor activation. These results indicated a direct coupling of receptor activation to a change in the spatial disposition of H3 and H6. This could occur if movements of H3 and H6 were coupled to changes in the conformation of the connected intracellular loops, which are known to contribute to binding surfaces and tertiary contacts of Rho with Gt (68, 69).

More direct evidence for changes in interhelical interactions upon receptor activation were provided by extensive site-directed spin labeling and EPR spectroscopy studies of the transition of Rho to R* in modified, or expressed mutant pigments. The results suggested a requirement for rigid body motion of transmembrane helices, especially H3 and H6, in the activation of Rho (65). A slight reorientation of helical segments upon receptor activation is also supported by experiments using polarized attenuated total reflectance infrared difference spectroscopy (46). Finally, movement of H6 was also detected by site-specific chemical labeling and fluorescence spectroscopy (51). The structural rearrangement of helices upon activation might not result in an R* structure that is drastically different from that of Rho since an engineered receptor with four disulfide bonds (between the cytoplasmic ends of H1 and H7 and H3 and H5, and the extracellular ends of H3 and H4 and H5 and H6) was still able to activate Gt (235).

Because the arrangement of the seven transmembrane segments is likely to be evolutionarily conserved among the family of GPCRs, the proposed motions of H3 and H6 may be a part of a conserved activation mechanism shared among all receptor subtypes (74, 110). In other class A GPCRs, agonist ligand binding would be coupled to a change in the orientations of H3 and H6. However, it should also be noted that the helix movement model of GPCR activation has been demonstrated so far only in the class A GPCR family. The recent crystal structure of the extracellular amino-terminal domain of a metabotropic glutamate receptor (mGluR) indicates that ligand-mediated receptor dimerization most likely plays a role in the activation mechanism (132). Other GPCRs in the class C family include the GABA receptor, the cal-
cium-sensing receptor, and some pheromone receptors. Currently, the activation mechanism in the class B GPCRs, including the glucagon receptor and related peptide receptors, is not clear (250). It remains likely in both class B and C receptors that agonist binding, which requires the NT, causes TM helix movement to transmit a signal across the membrane.

Some conformational changes must occur at the cytoplasmic surface of Rho to produce R* that can activate G. Does the Rho structure provide any potential insights that might help to predict the identity of these conformational changes? Does H8 unwind or come off the membrane surface activation? Do C2 and C3, which are known to be important for G activation, move? What are the active site conformations of the conserved Glu-134/Arg-135/Tyr-136 in H3 and the conserved Asn-302/Pro-303/Val-304/Ile-305/Tyr-306 in H7?

Synthetic Rho-derived peptides have been shown to compete with native Rho for G binding (129, 155). This has allowed the identification of the C2, C3, and the proximal portion of CT as G binding sites. Site-directed mutagenesis has further characterized groups of amino acids in these regions implicated in G binding and activation (55, 68, 69, 155). Mutagenesis experiments can be designed to elucidate the light-dependent alterations of physical or chemical states of specific amino acids required for G activation. For example, structural changes in the cytoplasmic surface domain of Rho were suggested by changes in the reactivities of Cys residues introduced at various positions by site-directed mutagenesis (123). Conformation-dependent interhelical interactions and tertiary contacts on the cytoplasmic surface were also probed biochemically using site-directed disulfide bond formation (234) and/or expression of split receptors (264, 265). With the use of the core of Rho as a scaffold, cytoplasmic loops of other GPCRs were substituted for those of Rho; the results of G protein activation experiments suggested that C2 and C3 might have distinct roles in G activation and G protein subtype specificity (261). These results also indirectly support the general activation mechanism of helix movement that transmits a signal from the membrane-embedded cored to the surface loops of the receptor.

Additional biophysical methods have been employed to probe conformational changes at the cytoplasmic surface domain concomitant with receptor activation. FTIR spectroscopic determination of protonation states or hydrogen bonding of specific amino acid side chains on the cytoplasmic surface of Rho is less advanced than for residues in the hydrophobic core of the receptor. This is in part due to the relatively small contribution of water-exposed amino acids to infrared absorption changes in typical FTIR samples (hydrated films) (70). Recently, attenuated total reflectance (ATR) FTIR difference spectroscopy of the R*-G (or peptides derived from G) complex revealed an infrared difference band that could be assigned to protonation of Glu-134 (56, 61, 183).

Other techniques have been successful in monitoring light-dependent structural alterations occurring during meta-II formation as well. As measured by hydrogen-deuterium exchange of buried peptide groups using time-resolved FTIR spectroscopy, the kinetics of exchange in R is increased. This result implies that buried regions of the peptide backbone structure become more accessible to solvent in forming meta-II (210). Light-dependent intramolecular charge movements in Rho can be monitored kinetically in vivo by recording an electroretinogram (ERG). The early phase of the ERG represents electrostatic potential in the oriented photoreceptor molecule. In fact, Rho displays a light-induced early receptor potential (ERP) akin to “gating currents” in voltage-dependent ion channels. Remarkably, the ERP can be recorded directly in giant cells that heterologously express Rho (223, 240). This method in combination with site-directed mutants may be able to identify specific amino acid side chains that move or exchange protons during the Rho photocycle.

Extensive site-directed spin labeling and EPR spectroscopy have been employed over the past 5 years to map the cytoplasmic domain of Rho (6, 33). EPR can provide information about side chain mobility, secondary structure, and solvent accessibility in both Rho and meta-II. Ultimately, this information can be assembled to form a map of structural changes that accompany receptor activation. Time-resolved (63) and static EPR spectroscopy studies (211) on site-specific spin-labeled Rho showed that the cytoplasmic terminations of H3 and H7 undergo structural rearrangements in the vicinities of Cys-140 and Cys-316, respectively. These changes have been specifically assigned to the meta-II conformation. Cys-140 is close to the highly conserved Glu-134/Arg-135/Tyr-136 tripeptide at the cytoplasmic border of H3.

Site-directed spin labeling of the amino acid residues from Tyr-306 to Leu-321 was also carried out. The information obtained regarding conformational changes in H8 upon meta-II formation was limited by the relative lack of reactivity of the Cys residues engineered into positions 317, 318, 320, and 321. However, structural changes were detected at positions 306, 313, and 316, consistent with movements of the nearby H6 and with biochemical evidence for a light-dependent interaction between Cys-65 and Cys-316 in native Rho (4, 32). Consistent with the notion of light-dependent structural changes in the vicinity of the cytoplasmic end of H7 is the observation that a monoclonal antibody with an epitope that was mapped to the amino acid sequence 304–311 bound only to R* and not to Rho (1). Only relatively small light-dependent structural changes were noted in and around the C1 loop when residues 56–75 were individually probed by site-directed spin labeling (5).
An EPR study of Rho mutants with a substitution of Glu-134 showed that the mutant receptors displayed an EPR signature consistent with a partially activated conformational state in the dark (118). This finding seems to be consistent with extensive earlier studies of Glu-134 replacement mutants. The structural change detected by EPR spectroscopy may be directly related to the apparent requirement for protonation of Glu-134 upon R* formation. A rearrangement of neighboring hydrogen-bonding partners may be necessary for protonation of Glu-134 to occur. The pH profile of Gt activation (59) as well as the abolishment of the uptake of two protons in mutant E134Q (9) suggests the existence of other titratable groups influenced by Glu-134. Glu-134 interacts primarily with Arg-135 in Rho, but it is not clear whether Glu-134 would interact with other side chains in R*, or simply be in a position to interact with bound Gt. The Glu-134/Arg-135 dipeptide may form a functional microdomain that is responsible for inducing the release of GDP from R*-bound Gt (2, 147).

Any model of receptor activation has to account for the fact that the chemical environment of the Schiff base is altered so that net proton transfer occurs between the Schiff base imine and Glu-113 (104). This change in the RET binding pocket must be transmitted to the cytoplasmic surface of the receptor. The Rho crystal structure is consistent with the helix movement model of receptor activation (65, 228) since it provides a structural basis to explain how chromophore isomerization could lead to displacement of H3 and H6 that would subsequently result in a change in orientation of Glu-134 at the cytoplasmic border. The structure suggests possible contacts between the cyclohexenyl ring of the chromophore and H3, which should change upon photoisomerization (26). At the Schiff base end of the chromophore, the C20 methyl group seems to interact with Trp-265, and this interaction should also change upon isomerization. Other key interhelical constraints are expected to be directly sensitive to chromophore isomerization, including those mediated by Phe-294, Ala-299, Asn-302, and Tyr-306. Any concerted disruption of stabilizing interhelical interactions may be expected to lead to helix movement and rearrangement of the helical bundle.

V. THE RHODOPSIN PHOTOCYCLE AND THE CHROMOPHORE REGENERATION PATHWAY

As outlined above, photoisomerization of the RET chromophore induces the active receptor conformation R* from the inactive conformation Rho (30, 192, 263). R* is spectroscopically indistinct from the Rho photoprodut meta-II, which is characterized by an unprotonated Schiff base chromophore linkage and a \( \lambda_{\text{max}} \) value of 380 nm. At low temperature, a number of photointermediates that characterize the transition of Rho to R* can be trapped and studied by a variety of spectroscopic techniques. The classical photolysis pathway of Rho at low temperatures proceeds according to the following scheme: Rho (500 nm) → bathorhodopsin (batho) (540 nm) → lumirhodopsin (lumi) (497 nm) → meta-I (478 nm) → meta-II (380 nm).

Laser flash photolysis coupled with nanosecond time-resolved UV-visible spectroscopy has identified a related photocycle that occurs at or near physiological temperature (124): Rho (500 nm) → batho (543 nm) → blue-shifted intermediate (BSI) (477 nm) → lumi (497 nm) → meta-I (480 nm) → meta-II (380 nm). Meta-II decays to metarhodopsin III (meta-III) (450 nm) and finally to opsin plus free 11-trans-retinal (141). The chromophore photoisomerization occurs on an ultrafast time scale and was observed to be a vibrationally coherent process (255). The Rho photocycle is summarized in Figure 6.

Significant progress has been made in understanding structure-function relationships in the photointermediates of Rho through optical, resonance Raman, FTIR, NMR, and other spectroscopic studies on a variety of native visual pigments, chemically modified pigments, and artificial pigments (native opsins reconstituted with synthetic retinal analogs; Refs. 25, 57, 62, 67, 89, 122, 124, 142, 149, 151, 196, 203, 224, 229). These types of studies provide specific detailed information about chromophore structures and about dynamic chromophore-opsin interactions.

In addition to probing the chromophore, specific absorbance changes by aromatic residues can also be measured in early Rho photointermediates (140). Trp-265 is a useful probe of specific chromophore-protein interactions during the Rho photocycle, especially given its proximity to the RET chromophore. It can be probed by UV absorption spectroscopy, fluorescence spectroscopy, or UV resonance Raman spectroscopy (126). The photocycle can also be studied under a variety of conditions that might provide a basis for identifying specific amino acid residues that might be involved in intramolecular proton transfer reactions. For example, the pH dependency was determined for the formation of Rho photoproducts from lumi to meta-II (109). These types of experiments could be carried out with site-directed mutants as well in future studies.

R* becomes phosphorylated by Rho kinase, and its ability to activate Gt is diminished by the binding of arrestin to phosphorylated R*. The RET Schiff base linkage also becomes accessible to water in its R* conformation. Through an autocatalytic mechanism that involves Glu-113 (225), the RET Schiff base is hydrolyzed and all-trans-retinal (ATR) is released in the outer segment of the rod cell. ATR dehydrogenase and NADPH. The all-trans-retinal is transported out of the rod cell where it binds to the interphotoreceptor retinoid-binding protein (IRBP). IRBP carries the all-trans-retinal to the retinal pigmented epithelium (RPE) where the chromophore is
released and transported into the RPE cell. The all-trans-retinol (pro-R at C15) is palmitoylated by lecithin retinol acyl transferase (LRAT). Isomerohydrolase acts on the all-trans-retinyl ester to form 11-cis-retinol (pro-S at C15). The 11-cis-retinol is oxidized to RET by 11-cis-retinol dehydrogenase in the RPE cell endoplasmic reticulum lumen. The RET is released at the apical pole of the RPE cell where it binds to IRBP and is transported to the rod cell. The RET binds to the opsin apoprotein to regenerate a Rho pigment. The details of the chromophore regeneration pathway are presented elsewhere (206, 219).

How does the retinal chromophore enter or exit the membrane-embedded core of the opsin apoprotein? The extracellular surface domain is very well organized, with many half-buried hydrophobic cores as well as H-bond networks. Considering this fact and the hydrophobicity of RET, it seems reasonable to assume that the chromophore enters and exits the membrane-embedded core of the receptor via the membrane phospholipid bilayer. This implies that the chromophore would have to pass between two adjacent transmembrane helices. There is a much wider gap between H5 and H6 than between any of the other helix pairs, largely due to the numerous Phe residues along one face of H5. One or two of the Phe residues might be imagined to move back and forth to allow the ligand to enter or exit.

VI. INTERACTION OF R* WITH MOLECULES INVOLVED IN VISUAL TRANSDUCTION

Although studies of the photocycle intermediates of Rho provide insights into dynamic changes in protein conformation and RET-protein interactions, R* is defined
as the conformation that catalyzes guanine nucleotide exchange by Gt and propagates and amplifies the signal of photon capture. R* is the key to the biochemical amplification cascade of the vertebrate visual system (Fig. 7). R* is spectrally indistinct from the meta-II photoprotein of Rho. Meta-II displays a $\lambda_{\text{max}}$ value of 380 nm due in part to the presence of an unprotonated RET Schiff base chromophore linkage. It is now widely accepted that meta-II exists in two isospectral forms (meta-IIa and meta-IIb) that differ at least in their protonation states (9, 10). Whereas meta-IIa cannot bind to Gt, meta-IIb may be identical to R*.

The transfer and amplification of signal from R* to Gt involves specific interactions between the cytoplasmic surface domain of R* and at least two regions of Gt, the carboxy-terminal tail of the $\alpha$-subunit and the carboxy-terminal tail of the $\gamma$-subunit (73, 95, 119). In fact, the binding of Gt, or certain peptides derived from Gt, can stabilize R* (53, 120). The stabilization of R* by Gt is related to the so-called “GTP effect” originally described for hormone receptors in hepatocyte membrane preparations (216). The molecular basis of the GTP effect is that receptors are stabilized in their high-affinity agonist conformation by the binding of G proteins. The addition of excess GTP causes G protein dissociation, and receptors return to a low-affinity agonist binding conformation. R* is stabilized in its high affinity 11-trans chromophore-bound state by interaction with Gt.

**FIG. 7.** Role of Rho in the biochemical cycle of visual transduction. Key protein-protein interactions take place on the disk membrane of the photoreceptor rod cell. Photoisomerization of the RET chromophore to all-trans produces R*. R* interacts with Gt to catalyze guanine nucleotide exchange by Gt. The GTP-bound $\alpha$-subunit of Gt activates cGMP phosphodiesterase (not shown). Rod cGMP levels fall and a plasma membrane cGMP-gated cation channel closes to cause hyperpolarization of the rod cell. R* is turned off in $\leq 1$ s by phosphorylation by Rho kinase and binding of arrestin to the phosphorylated R*. R* also decays more slowly due to Schiff base hydrolysis and loss of all-trans-retinal, which is then reduced to all-trans-retinol by retinal dehydrogenase and NADPH. The biochemical cycle of chromophore regeneration is largely carried out in the retinal pigment epithelial cells. Protein phosphatase 2A can dephosphorylate phospho-opsin to produce opsin apoprotein. One key feature of the vertebrate visual system is that each Rho molecule is used only once.
Perhaps the most extensively studied receptor-G protein interaction is that of bovine Rho with bovine G\(\alpha\), which can both be purified readily from bovine retinas. Detailed biochemical and biophysical analysis of the Rho-G\(\alpha\) interaction has been aided by mutagenesis of the cytoplasmic domain of bovine Rho. Numerous Rho mutants defective in the ability to activate G\(\alpha\) have been identified (69). Several of these mutant receptors were studied by flash photolysis (68), light scattering (54), proton uptake assays (9), or photoregeneration assays (55, 120). The salient results of these studies were that C\(_2\), C\(_3\), and CT (especially in the region involving H8) were involved in Rho-G\(\alpha\) interaction. These findings were consistent with other approaches including peptide competition studies using native Rho (129). A class of interesting receptor mutants was also identified in which spectrally normal meta-II-like pigments were formed upon illumination. The photolyzed mutants bound G\(\alpha\) in the presence of GDP, but failed to release the bound G\(\alpha\) in the presence of GTP (54, 68). These results were consistent with the idea that G\(\alpha\) binding and G\(\alpha\) activation were discrete steps mechanistically, which could be uncoupled by specific amino acid substitutions on the cytoplasmic surface of the receptor.

A number of cytoplasmic proteins in addition to G\(\alpha\) are known to interact with Rho. These include Rho kinase, which phosphorylates R\* at specific serine residues (Ser-334, Ser-338, and Ser-343) (157, 184, 198). Reconstitution experiments suggest that light-dependent phosphorylation of Rho is catalyzed primarily by Rho kinase, and not by protein kinase C (185). Phosphorylation decreases the effective lifetime of R\*, but paradoxically shifts the meta-I-meta-II equilibrium toward meta-II (75). Signal quenching requires that arrestin binds to phosphorylated R\* to prevent it from activating G\(\alpha\). The role of the distal carboxy-terminal tail in the shut-off of the light signal in the rod cell was shown convincingly in a study using a transgenic mouse strain. The mice, whose rod cells expressed a Rho transgene with a truncated carboxy-terminal tail, were studied by electrophysiological techniques. Phosphorylation of the carboxy-terminal tail was required for termination of light-induced signaling (42). A summary of some engineered animal models used to study rod cell physiology is presented in Table 1.

A detailed discussion of the use of physiological methods to address questions related to the termination of the flash response and to adaptation mechanisms has been presented (14, 15). For example, it appears that light adaptation cannot depend on high levels of phosphorylation of nonbleached Rho in rod cells (23). Light adaptation is regulated by intracellular calcium concentration. Intracellular calcium concentration is high in the dark but low in light-adapted rod cells. Although it is likely that the change in intracellular calcium concentration is detected by several cytoplasmic calcium-binding proteins that display calcium-dependent conformational changes (e.g., recoverin or S-modulin, isolated from bovine and frog rods, respectively), the mechanistic role of calcium, at least in terms of its effect on Rho phosphorylation, is not yet understood (115).

The biochemical mechanisms of background and light adaptation are known to involve light-dependent changes in intracellular concentrations of calcium as well as cGMP. Calcium levels influence the rate of accumulation of R\* (133). However, the precise effects of changes of calcium and cGMP concentrations on the rate of recovery during adaptation remain to be determined (78). The details of the regulation of the biochemical activities of Rho kinase, phosducin, recoverin, G\(\alpha\), and cGMP phosphodiesterase and their exact roles in signal termination and signaling system recovery and adaptation also remain to be elucidated in detail.

VII. RHODOPSIN MUTATIONS AS A CAUSE OF HUMAN DISEASE

Defects in G protein-mediated signaling are involved in the pathogenesis of a number of human diseases, both acquired and congenital (64, 231). The specific defects are often not fully understood, but two common themes of molecular pathophysiology generally emerge. First, the signal transduction machinery may be present and in its proper cellular location, but not properly functioning. Second, some element of the machinery may have failed to be properly synthesized, processed, or transported. These two paradigms appear to apply to Rho in cases of congenital stationary night blindness and retinitis pigmentosa, respectively.

Retinitis pigmentosa is a group of hereditary progressive blinding diseases with variable clinical presentations (21). One form of the disease, ADRP, was linked to a mutation in the gene for Rho (50). This first mutation discovered to be linked to ADRP was at codon 23 and would result in the change of a Pro residue to a His residue in the NT of Rho. Over 70 sites of rhodopsin mutation have been reported subsequently in ADRP patients (22, 28, 34, 48–50, 76, 156, 163, 197, 202, 226, 241). Recently, enough detailed phenotypic information has also been collected to attempt to group genotypes according to disease classes (43, 163).

Several studies have been carried out in which site-directed mutant opsin genes corresponding to ADRP genotypes were prepared (113, 164, 243). When expressed in tissue culture, the mutant opsins display a heterogeneity of spectral properties, biochemical properties, and cellular transport behavior. Some mutants are defective in chromophore binding, others in cellular transport and insertion into the plasma membrane. However, some mutations have no apparent effect in vitro. One particularly
interesting mutation linked to retinal degeneration is a replacement of the Schiff base Lys-296 by Glu (K296E). This mutation should prevent chromophore Schiff base formation. Interestingly, the corresponding mutation in bovine Rho was shown to cause constitutive activity of the mutant opsin in G, activation assays in vitro (215).

Significant work in this area has continued using transgenic mouse technology to produce mouse models of retinal degeneration. A summary of recent transgenic animal models is presented in Table 1. There appears to be no early developmental defect involving the retina in RP mouse models, and the work has potential relevance in understanding the molecular and cellular pathophysiology of some forms of ADRP (48, 50, 113, 241, 243). Transgenic mice expressing the P23H mutation on the intradiscal domain of Rho have been characterized, and results indicate that retinal degeneration may be due to improper mutant opsin expression and cellular transport (188, 217). Other interesting transgenic mouse models, such as those corresponding to mutations of the conserved residues Val-343 or Pro-347, point to a defect in targeting of the defective gene product to the rod cell outer segment (242). Rho mutants with alterations or deletions of these residues near the carboxy-terminal tail bind RET to form a pigment. In response to light, they activate G, and are phosphorylated normally.

Further work is needed to clarify how the various Rho mutations associated with ADRP lead to photoreceptor cell death and to degeneration of the many cell layers of the retina. However, in the vast majority of cases it appears that the inherited single allelic mutation in the Rho gene allows the synthesis of a Rho protein that is in some way abnormal in structure such that its normal cellular processing or transport is prevented. Over time this defect leads to the activation of cellular apoptotic pathways that eventually result in rod cell death. The progression from rod cell death to retinal degeneration may involve local inflammatory reactions. The effects of nutritional vitamin A supplementation, at least in animal models, suggest a potential therapeutic benefit by stabilizing mutant opsins through increased chromophore availability (144).

Constitutive activity of mutant opsin G90D has also been implicated as a cause of congenital night blindness (207), a defect in scotopic vision in which the dark noise level of the rod cell is increased (230). The mutant pigment associated with congenital night blindness has been characterized in some detail (207, 269). A discussion of activating mutations of Rho and other GPCRs has also been reported (208). Other inherited mutations in the vicinity of the Schiff base in Rho might affect chromophore stability or cause constitutive activity without necessarily leading to rod cell apoptosis. Polymorphisms might also be expected to result in some heterogeneity in scotopic visual sensitivity among the general population.

VIII. CONCLUSION

The recent report of the crystal structure of bovine Rho provides a unique opportunity to address questions related to the structural basis of Rho physiology in the vertebrate visual transduction cascade. Over the past decade a remarkable amount of information about structure-activity relationships in Rho, and other GPCRs, has been obtained using techniques of molecular biology. The crystal structure of Rho also provides a chance to evaluate the quality of this information. Site-directed mutant pigments have been employed to elucidate key structural elements, the opsin-shift mechanism, and the mechanism of receptor photoactivation. Much of the past work on Rho was aided by reasonable molecular models based on projection density maps of the membrane-embedded domain of Rho obtained from cryoelectron microscopy of Rho in reconstituted bilayers.

Perhaps the biggest surprise in the crystal structure was the role of the extracellular surface domain in defining the RET binding pocket. Whether or not this feature carries over to other GPCRs remains to be determined. One particular advantage of studying Rho has been the opportunity to employ various spectroscopic methods, especially in combination with site-directed mutagenesis. Optical spectroscopy and resonance Raman spectroscopy are possible because of the presence of the RET chromophore, which is probed as a sensor of chromophore-protein interactions. Difference spectroscopy techniques, such as FTIR and UV-visible difference spectroscopy, make use of the chromophore as an optical switch. Over-expression of recombinant Rho allows a variety of biophysical methods to be used to address particular questions related to protein and chromophore conformational changes. The recent use of engineered animal models to study not only rod cell development and cellular pathophysiology but structure-physiology relationships as well has been unusually productive.

The important work of the future will involve questions related to the precise molecular mechanism of signal transduction by Rho. This will require some understanding of the dynamic changes in protein conformation, not only in Rho, but in the other proteins of the vertebrate visual cascade.

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