

LESSONS FROM RHODOPSIN

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1. INTRODUCTION	85
2. METHODS	87
A. Preparation of Crude ROS Membranes	88
B. Preparation of UW-ROS and Solubilized Rhodopsin	89
C. Purification of Retinal Holotransducin and Transducin Subunits	89
D. Transducin Activation Assays	91
E. ROS Binding Assay	92
3. CHROMOPHORE-PROTEIN INTERACTIONS IN RHODOPSIN	93
4. MOLECULAR MECHANISM OF RHODOPSIN PHOTOACTIVATION	95
5. RHODOPSIN COUPLING TO TRANSDUCIN	97
6. CONSTITUTIVE ACTIVITY OF MUTANT OPSINS	98
7. STRUCTURAL MODELS OF RHODOPSIN	99

I. INTRODUCTION

Visual pigments comprise a large segment of the super-family of G protein-coupled receptors (GPCRs) (Sakmar, 1994, 1998). Since the molecular cloning of the gene for bovine rhodopsin in 1983 (Nathans and Hogness, 1983), a remarkable amount of information about structure-function relationships in GPCRs has been obtained using techniques of molecular biology. In the study of visual pigments in particular, site-directed mutagenesis has been employed to elucidate key structural elements, the opsin-shift mechanism, and the mechanism of receptor photoactivation.

One particular advantage of studying rhodopsin has been the opportunity to employ various spectroscopic methods in combination with site-directed mutagenesis. Optical spectroscopy and resonance Raman spectroscopy are possible because of the presence of the retinal chromophore, which is probed as a sensor of chromophore-protein interactions. Different spectroscopy techniques, such as Fourier-transform infrared (FTIR) and ultraviolet (UV)-visible difference spectroscopy, make use of the chromophore as an optical switch. Overexpression of

recombinant rhodopsin also allows a variety of biophysical methods to be used to address particular questions related to conformational changes and protein-protein interactions. Molecular models based on the two-dimensional projection structure of rhodopsin have also proven useful.

In summary, it is clear that information about rhodopsin structure and the molecular mechanism of rhodopsin photoactivation are relevant to understanding the molecular mechanism of signal transduction by GPCRs in general. This chapter focuses on recent technical and methodological advances in the study of recombinant rhodopsin that may be relevant to understanding the structure and function of GPCRs. Background information related to this chapter can be found in recent reviews (Fahmy and Sakmar, 1995; Sakmar, 1994, 1998).

Although they share many similarities with other GPCR types, there is significant specialization in visual pigments not found in other receptor families. In particular, pigments are made up of opsin apoprotein plus chromophore, 11-*cis*-retinal (Fig. 5.1). The chromophore is a cofactor and not a ligand in the classic sense because it is linked covalently via a protonated Schiff base bond to a specific lysine (Lys²⁹⁶) residue in the membrane-embedded domain of the protein. An important structural feature of the retinal chromophore in rhodopsin, in addition to its Schiff base linkage, is its extended conjugated polyene structure, which accounts for its visible absorption properties and allows for resonance structures (Rando, 1996). Rhodopsin has a broad visible absorption maximum (λ_{\max}) at about 500 nm. Photoisomerization of the 11-*cis* to all-*trans* form of the retinylidene chromophore is the primary event in visual signal transduction, and it is the only light-dependent step. Upon photoisomerization of the chromophore, the pigment is converted to metarhodopsin II (MII) with a λ_{\max} value of 380 nm. The MII intermediate is characterized by a deprotonated Schiff base chromophore linkage. MII is the active form of the recep-

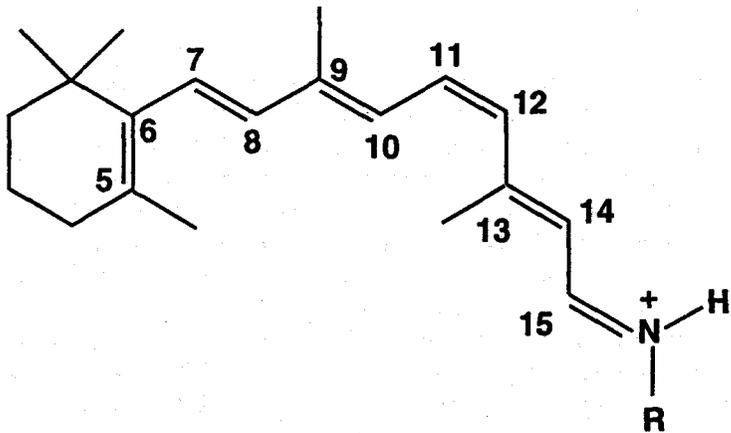


Figure 5.1. The chromophore of nearly all vertebrate visual pigments is 11-*cis*-retinylidene imine. The chromophore is covalently linked as a cofactor to Lys²⁹⁶ on transmembrane (TM) helix 7 via a protonated Schiff base bond. Photoisomerization of the 11-*cis*-retinylidene chromophore to the all-*trans* form is the only light-dependent event in vision. The numbering of the carbons in the conjugated polyene system is given. R signifies opsin.

tor (R^*), which catalyzes guanine-nucleotide exchange by the heterotrimeric G protein of the rod cell, transducin. In the case of the vertebrate visual system, GTP-bound transducin activates a cGMP phosphodiesterase, which lowers cGMP levels to close cGMP-gated cation channels in the plasma membrane of the rod cell. Light causes a graded hyperpolarization of the rod cell. The amplification, modulation, and regulation of the light response is of great physiological importance and has been discussed in detail elsewhere (Chabre, 1985; Stryer, 1991).

Bovine rhodopsin is the most extensively studied GPCR. A large amount of pigment (~ 0.5 mg) can be obtained from a single bovine retina by a sucrose density gradient centrifugation preparation of the rod outer segment (ROS) disc membranes. The pigment can be further purified by lectin affinity chromatography on concanavalin-A Sepharose resin. Rhodopsin is stable in solubilized form in a variety of detergents, including digitonin, dodecyl maltoside, and octyl glucoside. Rhodopsin was the first GPCR to be sequenced by amino acid sequencing (Ovchinnikov, 1982; Hargrave et al., 1983) and the first to be cloned (Nathans and Hogness, 1983, 1984). The cloning of the β_2 -adrenergic receptor (Dixon et al., 1986) led to the identification of the structural homologies that now define the large family of GPCRs.

2. METHODS

The bovine retina has been the tissue of choice for the study of the biochemistry of vertebrate visual signal transduction due to the unique histology of the rod cell and to the fact that $\sim 95\%$ of photoreceptor cells of the bovine retina are of the rod type. The relative ease with which ROSs, which contain all of the key components of the phototransduction system, can be isolated led to intense study by numerous investigators. More recent studies have been facilitated by the ability to express and to purify rhodopsin from transiently transfected mammalian cells in tissue culture (Oprian et al., 1987). Methods for the preparation of purified components of the bovine rod phototransduction system have been published, but over the course of our recent work a number of refinements were made, and several reported methods were combined to yield material appropriate for the biochemical reconstitutions required to assay recombinant receptors. This section presents methods for the preparation and biochemical assay of purified ROS membranes, holotransducin, and transducin α and $\beta\gamma$ subunits ($T\alpha$ and $T\beta\gamma$).

To carry out reconstitutions with rhodopsin-containing membranes, a method was developed to remove peripherally associated proteins from crude ROS membranes. Several extractions of the membranes were performed to yield a preparation of urea-washed ROS (UW-ROS) that contained nearly pure rhodopsin in oriented phospholipid disc membrane bilayers. Assays were developed using these membranes to demonstrate functional binding of transducin to rhodopsin and to measure the increase in the rate of nucleotide exchange of purified transducin in the presence of light-activated rhodopsin. Transducin activation was measured by determining the rate of nucleotide uptake of a nonhydrolyzable analog of GTP, GTP γ S. Time courses can be

measured by removing aliquots of a reaction and applying them to nitrocellulose filters under vacuum. Another assay of rhodopsin-dependent transducin activation was developed that takes advantage of the previous observation that the intrinsic tryptophan fluorescence of the α subunit of transducin increases dramatically when GTP rather than GDP occupies its nucleotide binding pocket. This assay provides a real-time measurement of GTP uptake by transducin and allows the calculation of kinetic rate constants that define discrete steps in the activation pathway.

2.A. Preparation of Crude ROS Membranes

The following procedure is based on a combination of methods from a variety of sources (Papermaster and Dreyer, 1974; Papermaster, 1982; Hong and Hubbell, 1973; Fung et al., 1981; Ting et al., 1993). Frozen retinæ were stored at -80°C until use. Buffers used were homogenizing buffer (HB): 42% (w/w) sucrose, 65 mM NaCl, 0.2 mM MgCl_2 , 5 mM HEPES, pH 7.5; dilution buffer (DB): 65 mM NaCl, 0.2 mM MgCl_2 , 5 mM HEPES, pH 7.5; and isolation buffer (IB): 10 mM MOPS, pH 7.5, 60 mM KCl, 30 mM NaCl, 2 mM MgCl_2 . All steps of the following procedure were carried out in a dark room illuminated only with dim red light (Kodak No. 1 safelight filters).

Vials of retinæ (4×50 retinæ/vial) were thawed in a water bath at 40° – 50°C . When the retinæ were partially thawed, they were transferred to a 600-ml beaker. HB (300 ml) was supplemented with soybean trypsin inhibitory protein (TIP) to 30 mg/ml, aprotinin to 10 mg/ml, PMSF to 0.1 mM, pepstatin to 0.7 mg/ml, and leupeptin to 10 mg/ml. The protease inhibitors can be excluded if preparing UW-ROS, but are necessary to prevent proteolytic degradation of transducin and cGMP phosphodiesterase. HB (120 ml) was added to the retinæ at room temperature, and they were stirred gently with a magnetic stirrer until completely thawed. The remaining steps were carried out on ice. The retinæ were divided among 8 50-ml polypropylene Sorvall SS-34 centrifuge tubes. Each tube was vortexed for 1 minute at high speed. This step breaks the ROS from the inner segment and releases it from the retinal tissue. In this solution, the specific gravity of the ROS causes them to float. The crude retinal extract was centrifuged in an SS-34 rotor at 4,000 rpm for 4 minutes at 4°C . The supernatant fractions were transferred to a 500-ml Erlenmeyer flask sitting on ice. Each pellet was resuspended in 15 ml HB (ice cold) and spun again. The resulting supernatant fractions were pooled with those of the previous spin in the flask. DB (300 ml) was slowly added to the membrane suspension while carefully swirling. Under these conditions, the membranes can be pelleted by gentle centrifugation. The membranes were divided again and spun in an SS-34 rotor at 4,000 rpm for 4 minutes at 4°C . The resulting supernatant fractions were carefully removed by aspiration and discarded.

The membranes are only loosely pelleted at this point. The supernatant fraction appears quite red in color due mainly to the presence of hemoglobin and not to the loss of rhodopsin-containing membranes. Each of the pellets was resuspended in 5 ml of 38% (w/w) sucrose in IB (IB-Suc) supplemented with TIP to 30 mg/ml, PMSF to 0.1 mM, and DTT to 1 mM. The resuspended pellets were pooled, and the volume was brought up to 250 ml with IB-Suc. Under these con-

ditions, the ROS membranes float, and contaminating membranes can be removed by centrifugation. The resuspended membranes were transferred to 10 50-ml SS-34 tubes and spun at 17,000 rpm for 15 minutes. The supernatant fractions were carefully transferred to a fresh 500-ml Erlenmeyer flask on ice, and 100 ml IB was added to the membrane suspension. The membrane suspension was transferred to six Beckman Ti-45 tubes and spun in an ultracentrifuge at 25,000 rpm for 15 minutes at 4°C. The supernatant fractions were removed by aspiration, and the membranes were resuspended in 120 ml IB, transferred to two Ti-45 tubes, and spun again. The pellets were resuspended again and spun as before. The ROS membranes can be stored overnight on ice until further processing of crude ROS to yield UW-ROS or transducin as described below.

2.B. Preparation of UW-ROS Membranes and Solubilized Rhodopsin

Crude bovine ROS membranes were prepared as described above. ROS membranes were then resuspended in wash buffer 1 (5 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 5 mM NaF, 30 mM AlCl₃) and stored overnight at 4°C. At each step, 0.5 ml of wash buffer was used per retina. ROS membranes were collected by centrifugation at 100,000g for 15 minutes, resuspended in wash buffer 1, and spun again. The membranes were then washed sequentially with wash buffer 2 (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT) three times, wash buffer 3 (200 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 1 mM DTT) once, wash buffer 4 (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 M urea) three times, and finally resuspended in assay buffer (10 mM PIPES, pH 7.5, 150 mM KCl, 4 mM Mg[OAc]₂, 0.1 mM EDTA, 1 mM DTT). The spectral ratio (A_{280}/A_{500}) of the UW-ROS is typically 1.8–2.0 measured after solubilizing the membranes in 1% (w/v) dodecyl maltoside. The concentration of rhodopsin was calculated using a molar extinction coefficient of 40,600 cm⁻¹ M⁻¹. The yield from 200 retinae is typically 3–4 mmol. Illumination of rhodopsin was carried out using a 150-W light source and a 495-nm long-pass optical filter (Melles-Griot). A photobleaching-difference spectrum can be calculated by subtracting the UV-visible spectrum of rhodopsin in the dark from that after illumination (Fig. 5.2).

2.C. Purification of Retinal Holotransducin and Transducin Subunits

Holotransducin was purified essentially as described elsewhere (Fung et al., 1981) from crude ROS prepared as described above. The membranes were illuminated for 5 minutes using a 150-W projector lamp fitted with a 495-nm long-pass filter and were then extracted with hypotonic buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF) two additional times. The fourth centrifugation was carried out at 30,000 rpm for 15 minutes and the fifth at 31,000 rpm for 15 minutes. The supernatant fractions from these two steps were discarded. Transducin was released from the membranes by two extractions with hypotonic buffer supplemented with 100 μM GTP. Both centrifugation steps were carried out at 42,000 rpm for 15 minutes. The released

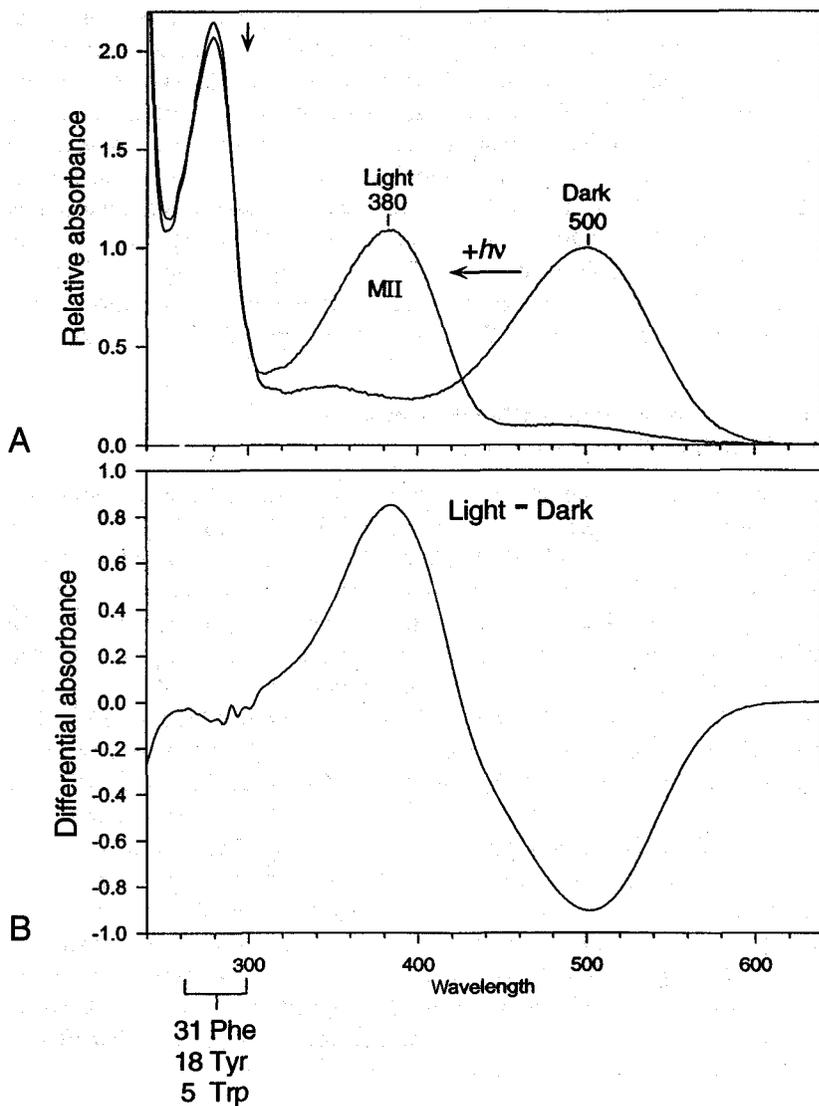


Figure 5.2. UV-visible spectroscopy of rhodopsin. **A:** UV-visible spectra in darkness and after illumination for purified rhodopsin in dodecyl maltoside (DM) detergent. Rhodopsin shows a characteristic broad visible absorbance with a λ_{\max} value of 500 nm. The 280-nm peak represents the protein component. Upon illumination in DM, the pigment readily forms the metarhodopsin (MII) intermediate with a λ_{\max} value of 380 nm. This spectral form of rhodopsin is the active conformation of the receptor (R^*), which catalyzes guanine-nucleotide exchange by transducin. Identical results can be obtained with rhodopsin from bovine retinae purified by concanavalin-A lectin affinity chromatography or with recombinant rhodopsin expressed in COS cells and purified by immunoaffinity methods. **B:** Photobleaching-difference spectrum obtained by subtracting the dark spectrum from the light spectrum in A. In addition to the large difference peaks noted at 380 and 500 nm, a light-dependent change in the opsin component of the spectrum is noted in the 260–300 nm range. This change is due to changes in the environments of aromatic residues as discussed in the text.

holotransducin was filtered through a 0.45-mm filter and loaded directly onto a hexyl-agarose (12-atom spacer, Sigma) column (1.0 × 9 cm) equilibrated in hexyl-agarose column buffer (HCB) (10 mM MOPS, pH 7.5, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF) overnight using a peristaltic pump set to 7.5 ml per hour. The peristaltic pump was reset to 0.5 ml per minute, and the column was washed with 60 ml of HCB. After a second wash with 75 mM NaCl in HCB, transducin was eluted from the column with a step gradient of 300 mM NaCl in HCB. Peak fractions were pooled and dialyzed against glycerol storage buffer (40% glycerol [v/v] in HCB supplemented with 10 mM GDP). The total yield of purified transducin was typically 3–8 mg from 200 retinæ. Purified transducin was stored at –20°C.

The subunits of transducin were prepared from purified holotransducin essentially as described (Shichi et al., 1984). A Blue Sepharose CL-6B (Pharmacia) column (1.5 × 9 cm) was equilibrated with Blue Sepharose column buffer (BSCB) (10 mM KH₂PO₄, pH 6.5, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 10% [w/v] glycerol) using a peristaltic pump set to 1.5 ml per minute. Purified transducin in glycerol storage buffer was diluted 20-fold in BSCB. Fractions (4.5 ml) were collected as the diluted transducin was loaded (fractions 1–9) onto the blue sepharose column and washed with 90 ml of BSCB (fractions 10–27). A 120-ml linear gradient from 0 to 2 M NaCl in BSCB was then applied to the column while collecting 2 ml fractions (fractions 28–72). Tβγ typically eluted early in the wash fractions, and Tα eluted in a broad peak in the middle of the NaCl gradient. Purified bovine transducin α (bTα) and transducin βγ (bTβγ) subunits were dialyzed against glycerol storage buffer and stored at –20°C.

2.D. Transducin Activation Assays

The filter-binding assay is based on the fact that transducin and its bound nucleotide are retained on nitrocellulose filters whereas free nucleotide passes through. The assay was carried out essentially as described elsewhere (Wessling-Resnick and Johnson, 1987; Min et al., 1993). Purified pigments were stored frozen at –20°C. Pigment concentrations were determined based on the A₅₀₀ value immediately before each assay. The assay mixture generally consisted of purified pigment (5 or 10 nM), purified transducin (7 μM) and ³⁵S-GTPγS (20 μM) in 0.1 ml of assay buffer (50 mM Tris-Cl, pH 7.2, 100 mM NaCl, 4 mM MgCl₂, 1 mM DTT, and 0.01% dodecyl maltoside). Time course experiments were begun in darkness at 10°C by the addition of nucleotide. After a 2-minute incubation, the assay mixture was illuminated continuously with a 150-W projector lamp (Dolan-Jenner) affixed with a 495-nm long-pass filter. At 30-second intervals in darkness and 15-second intervals during illumination, 10-μl aliquots were removed and transferred to the nitrocellulose filters. The filters were washed and dried, and bound nucleotide was quantitated using a Phosphor Imager System (Molecular Dynamics, Inc.) or by liquid scintillation counting. A time course in darkness was performed to rule out pigment dark activity or constitutive activity by mutant apoprotein present in the assay mixture. Control experiments in the absence of transducin revealed that less than 0.03% of the nucleotide bound to the filter.

Spectrofluorimetric assays of transducin activation can also be carried out to determine precise kinetic rate constants (Fahmy and Sakmar, 1993). The active transducin α -subunit concentration in a preparation of holotransducin can be precisely determined by measuring rhodopsin-catalyzed nucleotide-induced fluorescence increase upon addition of different amounts of GTP γ S (Fig. 5.3). The fluorescence assay employed was similar to one described elsewhere (Guy et al., 1990; Phillips and Cerione, 1988). Fluorescence was measured with a specially modified SPEX-Fluorolog II spectrofluorometer in signal/reference mode with excitation at 300 nm (2-nm band width) and emission at 345 nm (12-nm band width). Signal integration time was 2 seconds. The reaction mixture (1.6 ml) containing 10 mM Tris-Cl (pH 7.4), 100 mM NaCl, 2 mM MgCl₂, and 0.01% dodecyl maltoside, was cooled to 10°C and stirred continuously using a magnetic cuvette stirrer set at maximum speed. The addition of rhodopsin and nucleotide was done by injecting 50 μ l of the appropriate solution into the cuvette with a gastight syringe kept at 10°C. The sample was continuously illuminated in the cuvette with 543-nm light from a HeNe-laser (Melles-Griot) connected to the sample compartment by a fiberoptic light guide. Stray light was efficiently blocked from reaching the detector by a double monochromator. Under conditions of the assay, the rate of photobleaching to form a 380-nm species characteristic of MII was complete in 15 seconds.

2.E. ROS Binding Assay

The biochemical basis of this assay is the observation by Kuhn (1980) that transducin binds tightly to illuminated ROS membranes, even under hypotonic buffer conditions, and can be released by the addition of GTP. Varying amounts

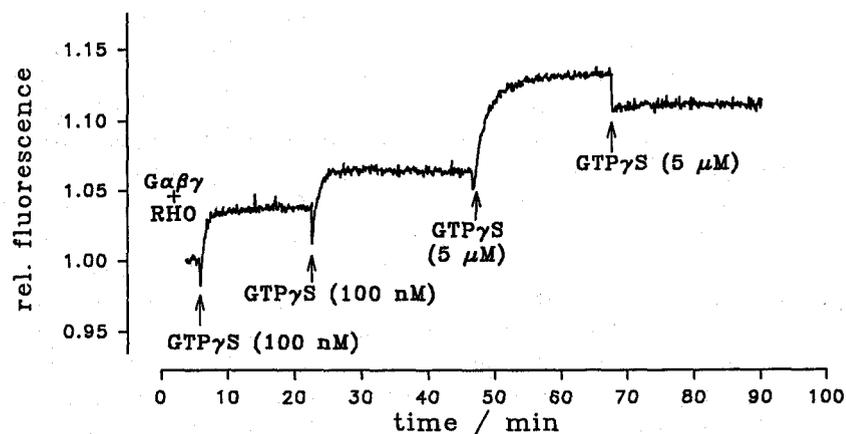


Figure 5.3. Spectrofluorometric titration of purified transducin with GTP γ S in the presence of purified COS-cell rhodopsin. Intrinsic tryptophan fluorescence of the α subunit of transducin is measured. GTP γ S binding by transducin, which is catalyzed by light-activated rhodopsin, causes a significant increase in fluorescence. The experiment is presented as a continuous time course with injections of GTP γ S into the sample cuvette indicated by arrows. The experimental details of the assay are presented in the text.

of purified transducin and UW-ROS (5 mM rhodopsin) were mixed for 15 minutes at 4°C in the dark in hypotonic buffer (5 mM HEPES, pH 7.5, 2 mM MgCl₂, 1 mM DTT). Each tube was illuminated for 5 minutes on ice using a 150-W light source affixed with a 495-nm long-pass filter. To estimate the amount of GTPγS binding activity present at the start of the reaction, an aliquot was removed from each tube and assayed. Membranes from each tube were washed by sequential centrifugation at 100,000g for 10 minutes and resuspended in hypotonic buffer twice. The washed membranes were then assayed for GTPγS binding activity. GTPγS binding activity before and after washing of the membranes was determined by the addition of ³⁵S-GTPγS (20 μM) and incubation for 1 hour at room temperature before application of three aliquots from each reaction onto nitrocellulose filters. The filters were washed and air dried, and the amount of radioactivity bound to the filters was determined by use of a Phosphor Imager System (Molecular Dynamics).

3. CHROMOPHORE-PROTEIN INTERACTIONS IN RHODOPSIN

Even though different visual pigments employ the same retinylidene protonated Schiff base chromophore, their λ_{\max} values span the visible spectrum from near UV at about 400 nm to far visible red at about 600 nm. Chromophore-protein interactions are responsible, directly or indirectly, for spectral tuning in visual pigments. Thus, differences in primary structure result in differences in spectral properties. The understanding of the molecular mechanism of spectral tuning has been advanced by the cloning of numerous visual pigments with different spectral properties. In many cases, primary structure alignments have led to testable hypotheses concerning the identities and mechanisms of key chromophore-protein interactions. For example, the cloning (Nathans et al., 1986) and *in vitro* spectral characterization (Oprian et al., 1991; Merbs and Nathans, 1992) of the human cone pigments has led to mutagenesis and Raman studies that have largely elucidated the mechanisms of green-red spectral tuning (Aseñjo et al., 1994; Kochendoerfer et al., 1997).

The chromophores in visual pigments lie within the helical bundle of the membrane-embedded domain of the receptor (Fig. 5.4). A number of studies have been carried out on bovine rhodopsin to investigate retinal-opsin interactions in the membrane-embedded domain of bovine rhodopsin. Several spectroscopic methods such as resonance Raman spectroscopy, FTIR-difference spectroscopy, and nuclear magnetic resonance spectroscopy have been employed (Birge, 1990; Siebert, 1995). Other approaches have included reconstitution of opsin apoprotein with synthetic retinal analogs (Honig et al., 1979) and photochemical crosslinking (Nakayama and Khorana, 1990; Zhang et al., 1994). Early work on the structure and function of recombinant bovine rhodopsin focusing on the use of techniques of molecular biology has been reviewed (Khorana, 1992; Nathans, 1992).

The environment of the Schiff base portion of the chromophore is a key protein-chromophore interaction in rhodopsin. Lys²⁹⁶ and Glu¹¹³ are the two amino acid residues that largely define the structure and function of the chromophore.

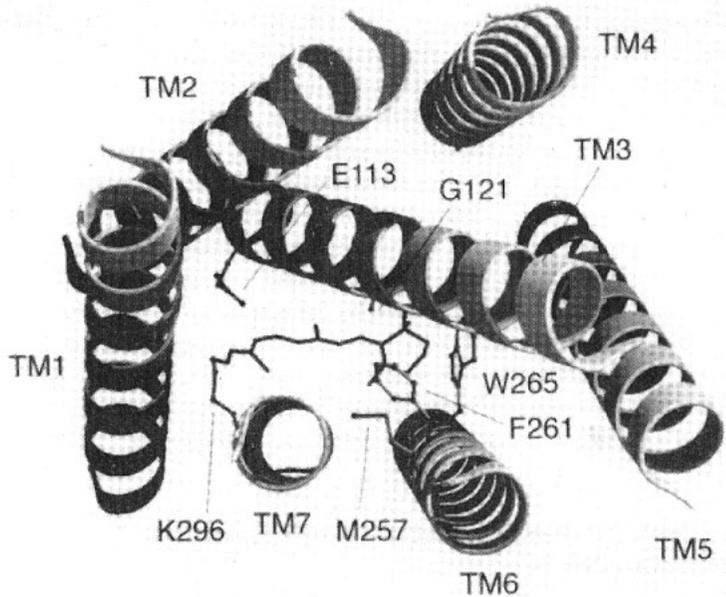


Figure 5.4. Molecular graphics model of the transmembrane (TM) domain bovine rhodopsin (Han et al., 1996b; Shieh et al., 1997). The molecule is viewed down the axis of TM helix 7 from above the plane of the disc membrane. TM helix 7 is thought to be roughly perpendicular to the membrane plane. TM helix 3 is tilted and buried within the bundle of TM helices. TM helices 3 and 6 largely define the retinal binding pocket, and key residues that interact with the chromophore include Glu¹¹³, Gly¹²¹, Phe²⁶¹, and Trp²⁶⁵. The retinal lies roughly at the level of Gly¹²¹ and Phe²⁶¹. Near the extracellular end of TM helix 3, Glu¹¹³ serves as the counterion to the protonated Schiff base localized near C₁₂ of the retinal. At the cytoplasmic end of TM helix 3 are three conserved residues (Glu¹³⁴, Arg¹³⁵, and Tyr¹³⁶, not shown), which are known to be involved in G protein binding. Movement of TM helices 3 and 6 in the plane of the membrane away from each other is thought to be required for receptor activation. An essential Cys¹¹⁰-Cys¹⁸⁷ disulfide bond at the extracellular surface of TM helix 3, which is highly conserved in GPCRs, might act as a pivot point for this motion and restrict vertical translation of the helix 3 relative to the other helices. The model of the opsin is based in part on data from electron microscopy studies of reconstituted bovine and frog rhodopsin. The positions and assignments of the TM helices are according to the projection map of the protein and a primary structure analysis of the GPCR family. The rationale for positioning the chromophore has been described elsewhere (Shieh et al., 1997). (Reproduced from Han et al. [1996b], with permission of the publisher).

The Schiff base linkage of the chromophore to Lys²⁹⁶ is known to be protonated in the ground state of the receptor (Hargrave et al., 1982). Light-dependent deprotonation of the Schiff base is required for the formation of R* (Kibelbek et al., 1991; Longstaff et al., 1986; Zhukovsky et al., 1991). However, light can induce R* in the absence of a Schiff base chromophore linkage in certain opsin mutants lacking a lysine at position 296 (Zhukovsky et al., 1991). Glu¹¹³ in bovine rhodopsin serves as the counterion to the positive charge of the protonated Schiff base (Nathans, 1990; Sakmar et al., 1989; Zhukovsky and Oprian,

1989). Glu¹¹³ is unprotonated and negatively charged in the ground state of rhodopsin (Fahmy et al., 1993). It becomes protonated upon light-dependent formation of MII and is the net proton acceptor for the Schiff base proton (Jäger et al., 1994). Glu¹¹³ is located near C₁₂ of the retinal polyene according to two-photon spectroscopy and nuclear magnetic resonance spectroscopy of retinal analogs and semiempirical quantum mechanical orbital calculations (Birge, 1990; Han et al., 1993; Han and Smith, 1995a,b). This location is supported by resonance Raman (Lin et al., 1992) and FTIR-difference spectroscopy studies (Fahmy et al., 1996) of recombinant mutant pigments lacking a carboxylic acid group at position 113.

The nature of the Glu¹¹³-protonated Schiff base interaction in the structure and function of rhodopsin has been elucidated indirectly by the discovery of constitutive activity among certain opsin mutants (Robinson et al., 1992; Rao and Oprian, 1996). Constitutive activity refers to the ability of an opsin to activate transducin in the absence of any chromophore. Generally, a mutation that disrupts the salt bridge between Glu¹¹³ and Lys²⁹⁶ in the opsin apoprotein leads to constitutive activity. For example, replacement of either Glu¹¹³ or Lys²⁹⁶ 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 affects the stability of the Glu¹¹³-Lys²⁹⁶ salt bridge (Rao et al., 1994; Cohen et al., 1992). The mechanism of constitutive activity of opsins and the potential relevance of constitutive activity to visual diseases such as congenital night blindness have been recently reviewed (Rao and Oprian, 1996). The concept of constitutive activity as it applies to GPCRs in general is discussed in more detail below.

4. MOLECULAR MECHANISM OF RHODOPSIN PHOTOACTIVATION

Photoisomerization of the 11-*cis*-retinal chromophore induces the active receptor conformation R* (Bownds, 1967; Dratz and Hargrave, 1983; Oseroff and Calender, 1974). Recent studies have suggested that steric and electrostatic changes in the ligand binding pocket of rhodopsin may cause changes in the relative disposition of TM helices within the core of the receptor (Shieh et al., 1997). These changes may be responsible for transmitting a "signal" from the membrane-embedded domain to the cytoplasmic surface of the receptor. Tryptophan mutagenesis (Han et al., 1996a,b; Lin and Sakmar, 1996), mutagenesis of conserved amino acid residues on TM helices 3 and 6 (Han et al., 1996a,b), and the introduction of pairs of histidine residues at the cytoplasmic borders of TM helices to create sites for metal chelation (Sheikh et al., 1996) have recently provided insights regarding the functional role of specific helix-helix interactions in rhodopsin. A series of elegant studies using site-directed mutagenesis of rhodopsin in combination with spin-labeling and electron paramagnetic resonance spectroscopy has revealed that the molecular mechanism of receptor activation after chromophore isomerization involves outward rigid-body movements of TM helices 3 and 6 relative to the center of the helix bundle (Farrens et al., 1996) (see also Chapter 13). Because the arrangement of the seven TM helices is

likely to be evolutionarily conserved among the family of GPCRs (Baldwin, 1993), the proposed motions of TM helices 3 and 6 may be a part of an activation mechanism shared among all GPCRs. In other receptor subtypes, ligand binding would have to be coupled to a change in the orientations of TM helices 3 and 6.

TM helix 3 of rhodopsin is known to be involved in chromophore-protein interactions and contains the retinal Schiff base counterion Glu¹¹³. Gly¹²¹, which is strictly conserved in all visual pigments, is located near the middle of TM helix 3. The α carbon of Gly¹²¹ was predicted to point toward the 9-methyl group of retinal based on a model of the chromophore orientation in the protein derived from solid-state nuclear magnetic resonance constraints (Han and Smith, 1995b). This model was tested using site-directed mutagenesis in combination with regeneration of mutant opsins with various chromophore analogs (Han et al., 1997a,b; 1998a). The results of these studies are consistent with the hypothesized orientation of the chromophore, which resides between Gly¹²¹ and Phe²⁶¹ (Fig. 5.4). Furthermore, these functional interactions between TM helices 3 and 6 mediated by the chromophore appear to be crucial for receptor photoactivation, which is mediated by movement of these two helices.

Synthetic rhodopsin-derived peptides have been shown to compete with native rhodopsin for transducin binding (König et al., 1989). This has allowed the identification of the cytoplasmic loops 3-4 and 5-6 and a putative loop between the cytoplasmic termination of TM helix 7 and the palmitoylated Cys³²² and Cys³²³ residues as transducin binding sites. Site-directed mutagenesis has further characterized groups of amino acids in these regions implicated in transducin binding and activation (Franke et al., 1990, 1992). Time-resolved (Farahbakhsh et al., 1993) and static electron paramagnetic resonance spectroscopy studies (Resek et al., 1993) on site-specific spin-labeled rhodopsin showed that the cytoplasmic ends of TM helices 3 and 7 undergo structural rearrangements in the vicinities of Cys¹⁴⁰ and Cys³¹⁶, respectively. These changes have been specifically assigned to the MII conformation. Cys¹⁴⁰ is close to the highly conserved Glu or Asp/Arg/Tyr triad at the cytoplasmic border of TM helix 3 (position 134-136 in bovine rhodopsin), which attracted attention in earlier studies because of its possible general importance for the function of GPCRs.

Replacement of Glu¹³⁴ by glutamine renders the photoactivated pigment about eightfold more efficient in activating transducin at alkaline pH than recombinant native rhodopsin (Fahmy and Sakmar, 1993). Therefore, it was suggested that Glu¹³⁴ is a good candidate for regulation of the transducin-binding region and may undergo a light-induced transition from an ionized to a protonated state (Fahmy and Sakmar, 1993; Cohen et al., 1993). Recent measurements of light-induced pH changes in the bulk-water phase monitored simultaneously with R* formation of the mutants E134Q and E134D showed the involvement of Glu¹³⁴ in proton uptake reactions (Arnis et al., 1994; Arnis and Hofmann, 1993). According to these results, it is likely that Glu¹³⁴ itself is a group that becomes protonated in MII. It has also been proposed that MII exists in two discrete conformations termed MII_a and MII_b, which are indistinguishable spectroscopically (Arnis and Hofmann, 1993). MII_b activates transducin and MII_a does not. It was proposed that one difference between these two forms of MII was the protonation state of Glu¹³⁴, where Glu¹³⁴ becomes protonated during the MII_a to MII_b transition.

The structural change detected by electron paramagnetic resonance spectroscopy may be directly related to protonation of Glu¹³⁴, which is expected to significantly alter the hydrogen-bonding properties of this amino acid. A rearrangement of neighboring hydrogen-bonding partners may then explain the conformational change. Indeed, electron paramagnetic resonance spectra of Glu¹³⁴ mutants showed that the receptor was in a partially active conformation (Kim et al., 1997). A possible functional importance of the relative positions of TM helices 3 and 4 of rhodopsin is also suggested on the basis of a conserved disulfide bond (Cys¹¹⁰–Cys¹⁸⁷) between these helices on the extracellular receptor domain (Doi et al., 1990; Karnik et al., 1988; Karnik and Khorana, 1990), which stabilizes the MII conformation (Davidson et al., 1994). In terms of the chromophore, the suggested localization of light-induced sterical changes would be expected mostly to affect locations distal to the Schiff base linkage, which is situated at the interfaces of TM helices 2, 3, and 7 (Rao et al., 1994; Schertler et al., 1993; Baldwin, 1993).

5. RHODOPSIN COUPLING TO TRANSDUCIN

Perhaps the most extensively studied receptor–G protein interaction is that of bovine rhodopsin with transducin (Hofmann et al., 1995). Detailed biochemical and biophysical analysis of the R*–transducin interaction has been aided by mutagenesis of the cytoplasmic domain of bovine rhodopsin. Numerous rhodopsin mutants defective in the ability to activate transducin have been identified (Franke et al., 1992). Several of these mutant receptors were studied by flash photolysis (Franke et al., 1990), light scattering (Ernst et al., 1995), or proton uptake assays (Arnis et al., 1994). The salient result of these studies was that cytoplasmic loops 3–4 and 5–6 were involved in R*–transducin interaction. This finding was consistent with those from other approaches, including peptide competition studies (Arnis et al., 1994). Although relatively large segments of cytoplasmic loops are required for proper rhodopsin–transducin interaction (Franke et al., 1992), single amino acid substitutions within these domains can have dramatic effects on transducin activation. In addition, transducin binding and the activation of bound transducin were shown to be discrete steps involving different surface domains of the receptor (Franke et al., 1990). Mutant pigments with alterations of cytoplasmic loops 3–4 and 5–6 were characterized that formed spectrally normal MII-like photoproducts that bound transducin (Franke et al., 1990). However, the bound transducin was apparently defective in the release of GDP, which accounts for the block in pigment-catalyzed GTP uptake or GTPase activity by transducin observed when the mutant pigments were assayed (Ernst et al., 1995). These results were consistent with the idea that transducin binding and activation were discrete steps mechanistically, which could be uncoupled by specific amino acid substitutions on the cytoplasmic surface of the receptor.

The role of the carboxy-terminal tail of rhodopsin in the activation of transducin is less well defined. Although several biochemical studies have implicated the carboxy-terminal tail in transducin activation (Phillips et al., 1992; Takemoto et al., 1986), alanine-scanning mutagenesis failed to confirm that the

proximal portion of the tail was required (Weiss et al., 1994; Osawa and Weiss, 1994). Additional work is needed to elucidate any role, subtle or otherwise, of the carboxy-terminal tail of rhodopsin in transducin activation. In addition, an important consideration will be to map the sites of specific transducin subunit interactions on the cytoplasmic surface of rhodopsin. This work may be facilitated by both the high-resolution crystal structure of heterotrimeric transducin (Lambright et al., 1996) and the ability to produce, using a baculovirus expression system, purified recombinant heterotrimeric transducin and to reconstitute it with recombinant rhodopsin.

6. CONSTITUTIVE ACTIVITY OF MUTANT OPSINS

Constitutive activity of GPCRs, defined as signaling in the absence of ligand, has been recognized as an important feature of these receptors since it was first reported to occur in mutant α_{1B} -adrenergic receptors (Cotecchia et al., 1990). The relationship between constitutive activity and ligand binding and activation can be described by the two-state model of GPCR function first proposed by Lefkowitz et al. (1993) and described quantitatively by Leff (1995). This model of GPCR function also seems to apply to rhodopsin (Han et al., 1998b).

Certain receptors also have intrinsically high levels of basal activity, which may be important for their physiological function (Tiberi and Caron, 1994; Cohen et al., 1997). Increases in signaling in the absence of ligand can also result from overexpression of either the receptor (Bond et al., 1995) or the G protein (Burstein et al., 1997). Rhodopsin, which is responsible for dim-light vision, has evolved a unique mechanism to minimize basal receptor activity and thus dark "noise." The chromophore 11-*cis*-retinal, which acts as a potent inverse agonist in rhodopsin, is covalently bound to the receptor to assure extremely low receptor signaling in the dark.

Constitutive activity of GPCRs has been implicated in the molecular pathophysiology of a number of human diseases. The mutant receptors responsible for these diseases are characterized by gain of function *in vivo* and *in vitro*. A partial list of these diseases includes familial male precocious puberty caused by constitutive activity of the luteinizing hormone receptor (Shenker et al., 1993), hyperfunctioning thyroid adenoma caused by constitutive activity of the thyrotropin receptor (Parma et al., 1993), hypocalcemia caused by constitutive activity of the Ca^{2+} -sensing receptor (Pollak et al., 1994), and Jansen-type metaphyseal chondrodysplasia caused by constitutive activity of the parathyroid hormone/parathyroid hormone-related peptide receptor (Schipani et al., 1995). Furthermore, Kaposi's sarcoma-associated herpes virus GPCR, homologous to the human interleukin-8 receptor, stimulates cellular proliferation by constitutively activating the phosphoinositide-inositol triphosphate-protein kinase C pathway and qualifies the receptor gene as a candidate viral oncogene (Arvanitakis et al., 1997).

Rhodopsin mutants that are constitutively active *in vitro* have also been found in visual diseases (Rao and Oprian, 1996). Congenital night blindness is caused by the rhodopsin mutation G90D or A292E (Dryja et al., 1993; Sieving et al., 1995), and forms of autosomal dominant retinitis pigmentosa are caused

by the mutations K296E or K296M (Keen et al., 1991; Rim and Oprian, 1995). One of the differences between rhodopsin and other GPCRs is that the inverse agonist 11-*cis*-retinal, which is able to suppress the constitutive activity of the mutant opsins G90D (Rao et al., 1994) and A292E (Dryja et al., 1993), is present in cells containing the mutant receptors. The G90D and A292E mutant receptors exist, at least in part, in the inactive ligand-bound form rather than the constitutively active apo-receptor form. Interestingly, patients carrying the G90D mutation were shown to have an elevated absolute threshold for visual perception (Sieving et al., 1995). FTIR spectroscopy of expressed mutant pigment G90D also suggested a possible increased thermal barrier to light-dependent receptor activation (Fahmy et al., 1996). Rhodopsin mutants K296E and K296M, however, do not bind 11-*cis*-retinal due to the lack of the lysine for Schiff base attachment (Rim and Oprian, 1995; Robinson et al., 1992). Therefore, significant amounts of these mutant opsin apo-receptors may be present to activate transducin, independently of either light or ligand. Recently, retinal analogs were designed and synthesized that specifically inhibit constitutive activity of these opsin mutants (Yang et al., 1997).

Many of the activating mutations in GPCRs are located in TM helix 6 and in the cytoplasmic loop connecting TM helices 5 and 6 (van Sande et al., 1995; van Rhee and Jacobson, 1996; Porcellini et al., 1994). As discussed above, many activating mutations of rhodopsin disrupt a salt bridge between Glu¹¹³ in TM helix 3 and Lys²⁹⁶ in TM helix 7 (Rao and Oprian, 1996), which is not conserved in GPCRs in general. However, additional activating rhodopsin mutations have been reported on TM helix 6 as well at Met²⁵⁷ and Phe²⁶¹ in the same region where activating mutations in many other GPCRs have been identified. In particular, Met²⁵⁷ may form an important and specific interhelical interaction that stabilizes the inactive receptor conformation by preventing TM helix 6 movement in the absence of all-*trans*-retinal. This stabilizing interaction may occur between Met²⁵⁷ and the NPXXY motif in TM helix 7, which is highly conserved in GPCRs. These results further support the idea that rhodopsin and other GPCRs share a common mechanism of receptor activation that involves specific changes in helix-helix interactions.

7. STRUCTURAL MODELS OF RHODOPSIN

Bacteriorhodopsin (BR), the light-driven proton pump of the halophilic bacterium *Halobacterium halobium*, contains seven TM helical segments and a retinylidene chromophore linked via a Schiff base to a specific lysine residue on TM helix 7. Although these similarities to rhodopsin are intriguing, there is no apparent primary structural homology among BR and visual pigments, including rhodopsin. In addition, BR does not couple to G proteins and has very short cytoplasmic loops compared with those of rhodopsin. Nevertheless, many of the same methodologies developed for the study of BR have also been applied to the study of rhodopsin. In addition, some of the paradigms involving the photochemistry and biophysics of membrane proteins involved in energy transduction or vectorial transport can be applied to both systems (Lanyi, 1995).

BR forms an ordered two-dimensional lattice in the "purple membrane" of *H. halobium*, which has made it possible to undertake high-resolution structural studies. The reconstructed three-dimensional structural model of BR (Henderson et al., 1990) and its subsequent refinements provided a template for the superimposition of the seven TM segments of GPCRs (Röper et al., 1994), including rhodopsin (Lin et al., 1992). Despite the recent reports of high-resolution three-dimensional structures for BR (Pebay-Peyroula et al., 1997; Kimura et al., 1997), the lack of a clear evolutionary relationship between BR and rhodopsin or other GPCRs makes modeling approaches based on BR structures somewhat speculative.

Recently, cryoelectron microscopy and image reconstruction were employed to determine the projection structures at about 9 Å resolution of bovine rhodopsin reconstituted into phospholipid bilayers (Schertler et al., 1993). Higher resolution structures of frog rhodopsin were subsequently reported (Schertler and Hargrave, 1995; Unger et al., 1997). Helix assignments and rotational orientations were proposed for the projection densities based on a comparison of a large number of GPCR amino acid sequences and on relevant biochemical and mutagenesis studies (Baldwin et al., 1997). The helix assignments for rhodopsin are consecutive at the extracellular membrane surface. However, different tilt angles of the helices as they traverse the membrane result in TM helix 3 being much more central in location at the cytoplasmic membrane surface.

Detailed molecular modeling based on these projection maps of rhodopsin has proven informative, and integrated approaches, which consider a variety of information in addition to sequence alignments, have been developed (Herzyk and Hubbard, 1995; Pogozheva et al., 1997). An example of one such model of rhodopsin is shown in Figure 5.4 (Shieh et al., 1997). Such models have been used extensively in ligand binding site analyses with the aim of "rationale drug design." In addition, when reasonable models are combined with experimental data on receptor activation, including studies of recombinant mutant pigments, a tentative assessment of intramolecular processes that may be of functional importance during activation of rhodopsin, and of other GPCRs, might be attempted either empirically or by molecular dynamic simulations (Gether et al., 1997; Colson et al., 1998).

It should be noted that models based on the projection maps of rhodopsin generally only consider the membrane-embedded domain of the receptor. However, TM helices can be connected by loop structures predicted by using secondary structure prediction algorithms. Also, some three-dimensional structural information concerning the cytoplasmic surface of rhodopsin has been inferred by solving the solution structures of peptides corresponding to putative cytoplasmic domains. Multidimensional nuclear magnetic resonance (NOSEY) spectroscopy and circular dichroism were employed to study peptides corresponding to the third cytoplasmic loop (loop 5–6) and the carboxy-terminal tail of bovine rhodopsin (Yeagle et al., 1995, 1997). The loop 5–6 peptide seemed to fold to form a stable structure that could be docked onto the ends of the appropriate TM helices identified in the projection models (see Chapter 16).

ACKNOWLEDGMENTS

T.P.S. is an Associate Investigator of the Howard Hughes Medical Institute. Support was also provided by the Allene Reuss Memorial Trust. K.C.M. was supported in part by NIH MSTP grant GM 07739. We thank our collaborators on studies of visual phototransduction over the past 6 years: H. Bourne, K. Fahmy, S.G. Graber, K.P. Hofmann, D.S. Kliger, R.A. Mathies, K. Nakanishi, H. Ostrer, F. Siebert, and S.O. Smith.

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