I. Introduction

Advances in the study of photoreceptor cells have proceeded at a rapid pace during the past decade. Our increased understanding of cell functions and the interaction between the photoreceptor-rhodopsin and its G protein transducin (Gt) has benefited from and been driven by numerous biochemical and biophysical methods of analysis. Research on the rhodopsin/G_t-system has contributed substantially towards our understanding of G protein-coupled signal transduction. Exploring the mechanism of the interaction between rhodopsin, a prototypic GPCR and transducin is important for further pharmacological approaches aimed at rescuing the phenotype in eye diseases, and could also impact on the treatment of other GPCR-linked diseases.

1.1 Visual Signal Transduction

The retina of eyes comprises seven major classes of cells and around 50 cell types, which are extremely highly organized. Vertebrates have 2 types of photoreceptors: rods, which are very sensitive to light and used for vision in dim light (no color), and cones used for bright light and color vision. The human retina contains ~100 million rods and 3 million cones. Rods and cones convert the light signal into a nerve impulse. The synapse is in contacts with retinal bipolar cells and other cells. The retina processes information from the photoreceptor which is then transmitted to the brain by the optic nerve.

The rod photoreceptor cell is the most studied in terms of molecular genetics, protein structures and physiology. The structural and functional studies performed on this cell will form a rich source for interpretation of the new data that will soon become available for the cone cells.

1.1.1 Rod Cell

The rod cell plays a vital role in vertebrates visual signal transduction because its outer segment is specialized for photoreception. It contains a stack of about 1000 discs which are closed, flattened sacs with a thickness of 160 Å thick. These membranous structures are

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densely packed with photoreceptor molecules called rhodopsin. The disc membranes are separate from the plasma membrane of the outer segment (Fig.1). These discs are continually renewed and last a month. A slender immotile cilium joins the outer segment and the inner segment, which is rich in mitochondria and ribosomes. The inner segment generates ATP at a very rapid rate and is highly active in synthesizing proteins and it is next to the synaptic body. Many synaptic vesicles are present in the synaptic body, which forms a synapse with a bipolar neuron.

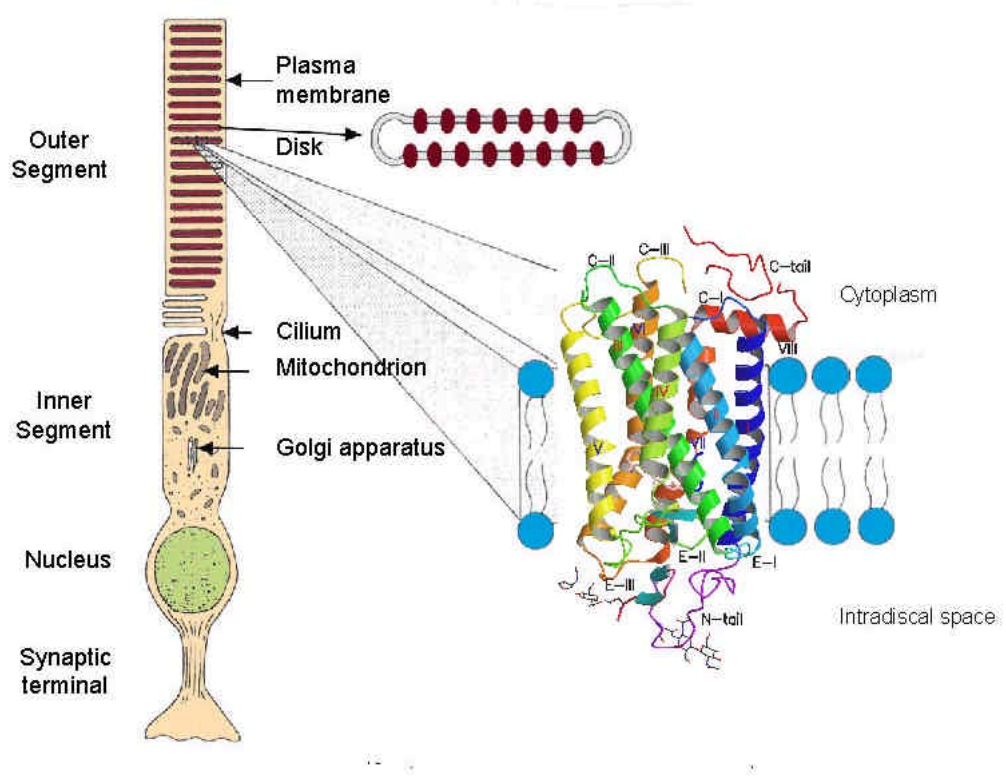

Fig. 1. The Vertebrate Rod Cell (adapted from Hargrave et al., 1993 and Palczewski et al**.,** 2000) Rod cell outer segments consist of stacks of disc membranes containing the photoreceptor protein rhodopsin. The expanded cross section of the disc membrane shows the oval shapes of densely packed three dimensional model rhodopsin molecules that traverse the lipid bilayer. To the front right of the figure is a drawing of the polypeptide chain of rhodopsin as it may exist in the lipid bilayer. The N-terminal exposes two attached oligosaccharide chains at the intradiscal surface. The chromophore ligand retinal is shown in pink and is buried in the seven helix

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1.1.2 Structure and Function of Rhodopsin

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 Family A GPCRs, which share primary structural homology (Sakmar, 1994 and Strader et al., 1994), i.e. the seven transmembrane segments. Rho can be obtained from bovine retinae (~0.5 mg/retina) by a sucrose density gradient centrifugation preparation of the rod outer-segment disc membranes (Papermaster et al., 1982). Rho is stable enough in the dark to be purified further by various chromatographic procedures, and it remains stable in solution in a variety of detergents (Okada er al., 1998). Bovine Rho was the first GPCR to be sequenced by amino acid sequencing (Hargrave et al., 1983 and Ovchinnikov et al., 1982), the first to be cloned (Nathans et al., 1983 and 1984), the first to be crystallized (Okada et al., 1998), and the first to yield a crystal structure (Palczewski et al., 2000).

Although it shares many similarities with other GPCRs, as a visual pigment, Rho displays many specialized features not found in other GPCRs. In particular, visual pigments are made of the opsin apoprotein plus the chromophore 11-*cis*-retinal (RET). The chromophore is not a ligand in the classical sense because it is linked covalently via a protonated Schiff base bond in the membrane-embedded domain of the protein. The Lys residue that acts as the attachment site for the chromophore (Lys-296) is conserved within rhodopsins and located in the transmembrane helix 7 (H7, Fig. 2) . A carboxylic acid residue that serves as the counterion to the protonated, positively charged retinylidene Schiff base (Glu-113) is conserved within H3 (reviewed by Sakmar, 2002).

1.1.2.1 Structure and Function of the Extracellular Surface Domain of Rhodopsin

The extracellular surface domain of Rho comprises the amino-terminal tail (NT) and three interhelical loops (EI, EII, and EIII) (Fig. 2). There is significant secondary structure in the

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extracellular domain and several intra- and interdomain interactions. NT extends from the amino terminus to Pro-34 and contains five distorted ß-strands. The extracellular surface domain also contains three extracellular interhelical loops: loop E1 (a.a. 101–106) connects H2 and H3, loop E2 (a.a. 174–199) connects H4 and H5, loop E3 (a.a. 278–285) connects H6 and H7.

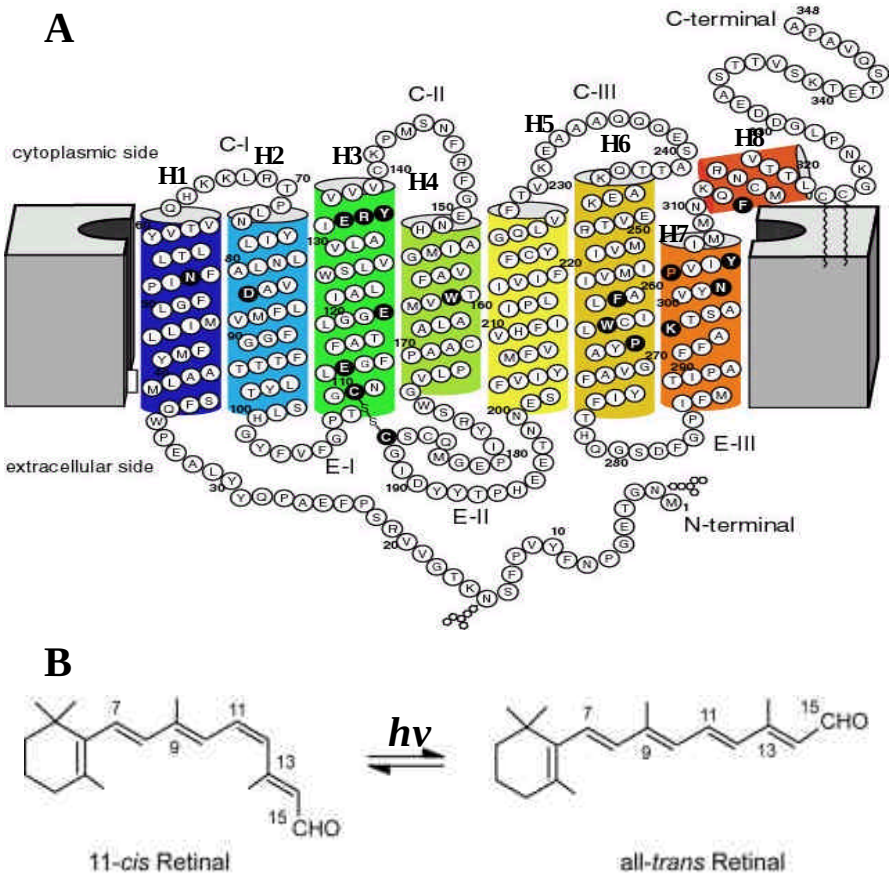

Fig. 2. Two-dimensional Model of Bovine Rhodopsin
(A) Secondary structure model of rhodopsin (Palczewski et al., 2000) showing the three domains: cytoplasmic, membrane-embeded, and intradiscal. Single-letter abbreviations are used for the amino acids. Rhodopsin's polypeptide chain traverses the lipid bilayer seven times as helical segments (H1 to H7). Hydrophilic loop sequences are exposed to the cytoplasmic surface (CI-CIII) and to the intradiscal or extracellular surface (EI-EIII). The amino-terminus contains oligosaccharide chains at N2 and N15. Retinal is linked via a Schiff base to K 296 in helix 7. Residues shown in black circles indicate the position of mutations associated with retinitis pigmentosa. In rhodopsin, extracellular corresponds to the intradiscal space.
(B) Isomerization of 11-cis-retinal to all-trans-retinal due to absorption of light.

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One of the most striking features of the Rho structure is the presence and positioning of the ß-strand containing Ser-186/Cys-187/Gly-188/Ile-189, which forms an extracellular roof for the RET-binding pocket (Fig. 5). These ß-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.

The NT of Rho is glycosylated at Asn-2 and Asn-15. It was concluded that glycosylation at Asn-15 was required for full signal transduction activity, but apparently not for correct biosynthesis or folding (Kaushal et al., 1994). The extracellular loops and amino-terminal tail 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 (Doi et al., 1990).

1.1.2.2 Structure and Function of the Membrane-embedded Domain of Rhodopsin

The membrane-embedded domain consists of seven transmembrane segments (H-1 to H-7), which are predominantly á-helical. 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. The 11-*cis*-retinal chromophore is bound in a pocket of this domain. All seven transmembrane segments and part of the extracellular domain contribute to interactions with the bound chromophore (reviewed by Menon et al., 2000).

The most striking feature of the RET binding pocket is the presence of many polar or polarizable groups to coordinate an essentially hydrophobic ligand. The Schiff base linkage of the chromophore to Lys-296 is a key feature of Rho structure (Hargrave et al., 1982). Glu-113 in bovine Rho serves as the counterion to the positive charge of the RET protonated schiff base (Nathans et al., 1983; Sakmar et al., 1989; Zhukovsky et al., 1989).

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Glu-113 is unprotonated and negatively charged in the ground state of Rho (Fahmy et al., 1993).

1.1.2.3 Structure and Function of Cytoplasmic Surface Domain of Rhodopsin

The cytoplasmic domain of Rho comprises three cytoplasmic loops and the carboxyl-terminal tail: CI (a.a. 65–70), CII (a.a. 140–150), CIII (a.a. 226–246), and CT (a.a. 307–348). The cytosolic side contains a region that transmits the signal to an enzymatic cascade. An overlapping region participates in deaction of the activated receptor. The phosphorylation of serine and threonine residues in the carboxyl-terminal tail is a step in the deactivation process. A number of cytoplasmic proteins are known to interact exclusively with activated rhodopsin (R*). Several of mutant receptors were studied by flash photolysis (Ernst et al., 2000; Franke et al., 1990), light scattering (Ernst et al., 1995), or proton uptake assays (Arnis et al., 1994). The key overall result of these studies is that CII, CIII and H8 are involved in R*-Gt interaction. Recently, a combination of site-directed mutagenesis and peptide binding studies clearly showed that the H8 region, is involved in Gt binding and activation (Ernst et al., 2000; Khorana et al., 1999; Marin et al., 2000). 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 expressed (Tai et al., 1999). Conformational changes in the cytoplasmic surface domain that are coupled to chromophore isomerization initiate the intermolecular transduction of the light signal in rods.

1.1.3 Signal Transduction in Vertebrate Photoreceptors

The initial event in visual excitation is light-absorption by rhodopsin which causes isomerization of 11-cis-retinal to all-trans-retinal (Wald et al., 1968). This leads to a conformational change from the inactive to the active form of meta rhodopsin II (MII, R*).

The light-activated rhodopsin can then bind and activate transducin (Gt) (Hamm et al.,

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1998 and 1996; Hofmann, 1999), by catalyzing the rapid exchange of GTP for bound GDP on the α-subunit of Gt (Gtα) in transducin. Binding of GTP causes dissociation of Gtα from Gtβγ and activates the membrane-associated cGMP-phosphodiesterase (PDE) (Heck and Hofmann, 1993). The activated PDE then hydrolyzes very rapidly cyclic GMP (to 5'-GMP). cGMP acts as a second messenger in the rod cell which transmits the signal to the plasma membrane. A decrease in the cGMP concentration then leads to the closure of cGMP-gated cation channels in the plasma membrane of the rod outer segment (Bourne et al., 1997). This causes a hyperpolarization of the plasma membrane which becomes more negative on the inside. The light induced hypolarization is then passively transmitted by the plasma membrane to the synaptic body (Fig. 3).

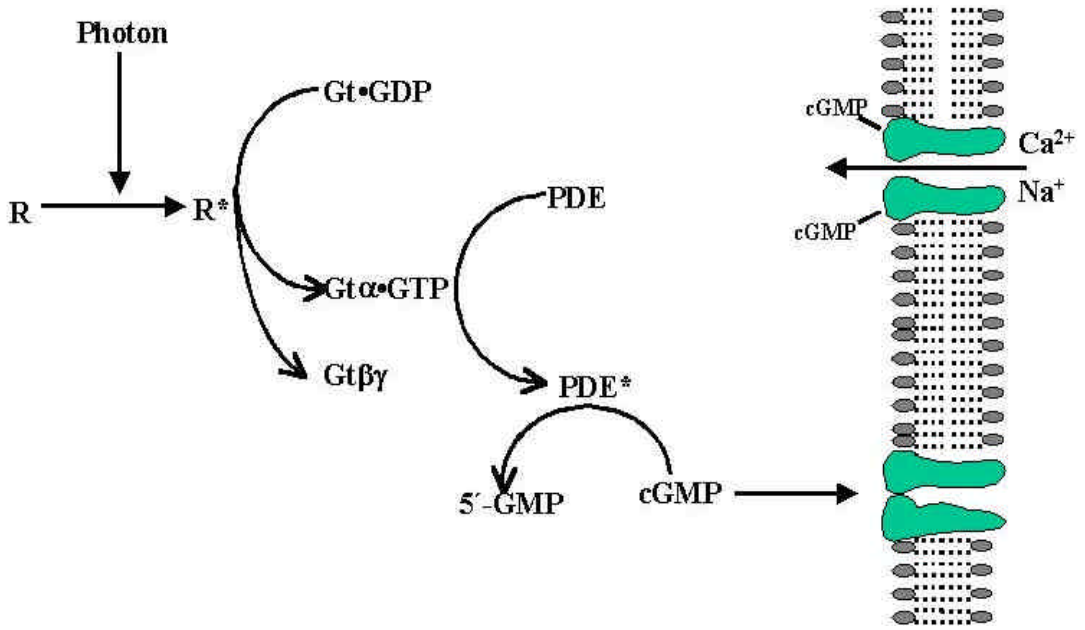

Fig. 3. Signal Transduction in Vertebrate Photoreceptors

1.1.4 Rhodopsin Photocycle

After activation, both retinal and the protein change their conformations, as reflected in the formation of a series of transient intermediates with distinct spectral properties. The Schiff

base linkage becomes deprotonated in the transition from metarhodopsin I to metarhodopsin II, the photoactivated state (R*). R* exerts its effect on rod cell biochemistry by catalyzing nucleotide exchange in Gt. To stop signal transduction, R* is then phosphorylated by rhodopsin kinase, which phosphorylates specific serines and threonines in rhodopsin's carboxyl-terminal sequence. This phosphorylation reduces the ability of transduction to interact with R*, but does not totally eliminate it. However, now arrestin can bind to the phosphorylated and photoactivated rhodopsin (R*P), and this totally prevents further activation of transducin. This complex eventually decays as the all-*trans*-retinal dissociates, and the phosphates are removed by a phosphatase (PrP2A). The all-*trans*-retinal is regenerated in a series of enzymatic steps to 11-*cis*-retinal, and the retinal rebinds to the protein opsin, regenerating rhodopsin (review of Hargrave et al., 1992) (Fig. 4).

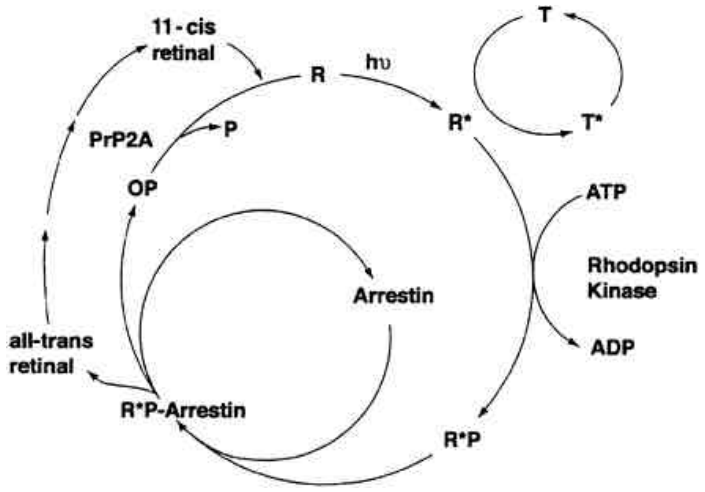

Fig. 4. The Rhodopsin Cycle (From Hargrave, 2000)

The chromophore photoisomerization occurs on an ultra fast timescale (Wang et al., 1994). 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. Laser flash photolysis coupled with nanosecond time-resolved UV-visible spectroscopy has identified the Rho photocycle that occurs at or near physiological temperature: Rho (500 nm)

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bathorhodopsin (543 nm) blue -shifted intermediate (BSI) (477 nm) lumirhodopsin (497 nm) MI (480 nm) MII (380 nm). MII decays to metarhodopsin III (MIII) (450 nm) and finally to opsin plus free all-*trans*-retinal (Lewis et al., 2000).

The photointermediates of Rho and a variety of native visual pigments, chemically modified pigments, and artificial pigments have been studied by optical, resonance Raman, FTIR, and NMR spectroscopy (Birge et al., 1985; Fahmy et al., 1993; Fahmy et al., 1995; Feng et al., 2000; Han et al., 1995; Klein-Seetharaman et al., 1999; Kliger et al., 1995; Lewis et al., 2000; Lin et al., 1996 and 1992; Palings et al., 1987; Popp et al, 1996; Sakmar et al., 1995; Siebert, 1995). These studies provided specific information about chromophore structures and about dynamic chromophore-opsin interactions. 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.

1.2. The Structural Basis of Rhodopsin-transducin Interactions

1. 2.1 Structural Studies of Transducin

Gt plays a central role in the phototransduction cascade (Stryer, 1988 and Wald, 1968). Gt couples together two separate highly specialized proteins: the photon detector rhodopsin and the efficient second messenger modulator PDE. Gt activation by R* represents a key amplification step in the cascade in that a single R* can catalyze the activation of hundreds of Gt molecules (Fung et al., 1983; Heck et al., 2001) In addition, Gt exhibits a low rate of basal (uncatalyzed) nucleotide exchange that contributes to sensitivity by maintaining low background noise. Finally, Gt provides an important site of regulation. The rates of GTP loading and GTP hydrolysis by Gt determine to a large extent the amplitude and the temporal resolution of the resulting signal.

Gtα, a 350–amino acid protein, consists of two domains (Fig. 5)—the Ras-like domain, so named because of its homology with the monomeric G protein, $p21^{ras}$ (Ras), and the helical

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domain, so named to reflect its composition of six α-helices (αA-αF). The nucleotide is bound in a cleft between the domains.

Gtα-GTP activates PDE, a tetrameric enzyme consisting of α, ß, and γ subunits in a 1:1:2 stoichiometry, by removing the inhibitory constraints that the γ subunits exert upon the catalytic α and ß subunits. The binding site of PDEγ on Gαt was recently determined by X-ray crystallography and found to reside between the α2-helix of the Switch II region and the adjacent α3-helix (Slep et al., 2001).

Gtα hydrolyzes bound GTP to return to its inactive GDP-bound state. Rapid turn-off of the cascade is essential for the temporal resolution of the signal (He et al., 1998). GTP hydrolysis is accelerated by the simultaneous binding of the PDEγ subunit, and a second protein, regulator of G protein signaling 9 (RGS9) (He et al., 1998). Gtα (GDP) recombines with Gtβγ and can then be activated again by another R*.

Gtß is a 340–amino acid protein, constructed from an amino-terminal α-helix, followed by a ß-propeller structure (Sondek et al., 1994). The ß-propeller consists of seven "blades," each consisting of four ß-sheets. Each blade is roughly related to the others by rotational symmetry. At the sequence level, Gßt is notable for seven WD40 domains, sequence repeats of roughly 40 amino acids that frequently end with Trp-Asp (WD in the single letter code). Each WD40 repeat corresponds to the fourth strand of one propeller blade and the first three strands of an adjacent blade.

Gtγ is the shortest Gt subunit, consisting of only 73 amino acids. It contains an amino-terminal α-helix, which interacts with the amino terminus of Gtß in a coiled-coil conformation (Fig. 5). The remainder of Gtγ wraps around Gtß in an extended conformation. Gtß and Gtγ can be dissociated from one another only under denaturing conditions, and physiologically they function as a single entity.

1.2.2 The Mechanism of Rhodopsin-Catalyzed Nucleotide Exchange

The crystal structures of heterotrimeric Gt (Lambright et al., 1996) have allowed the identification of subunit binding sites and provided a fundamental context for

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understanding how the G protein heterotrimer interacts with membranes and the activated receptor, and how GTP binding leads to subunit dissociation and effector activation (Medkova et al., 2002). The photoisomerization of 11-*cis*-retinal to all-*trans*-retinal (ATR) leads to local structural alterations in the chromophore-binding pocket of Rho. These structural changes are propagated to the cytoplasmic surface of Rho, and following binding of Gt, on to the nucleotide-binding pocket of Gtα where GDP is released. In this way, the chromophore-binding pocket of Rho is allosterically coupled to the nucleotide-binding pocket of Gtα approximately 5 nm away (Fig. 5).

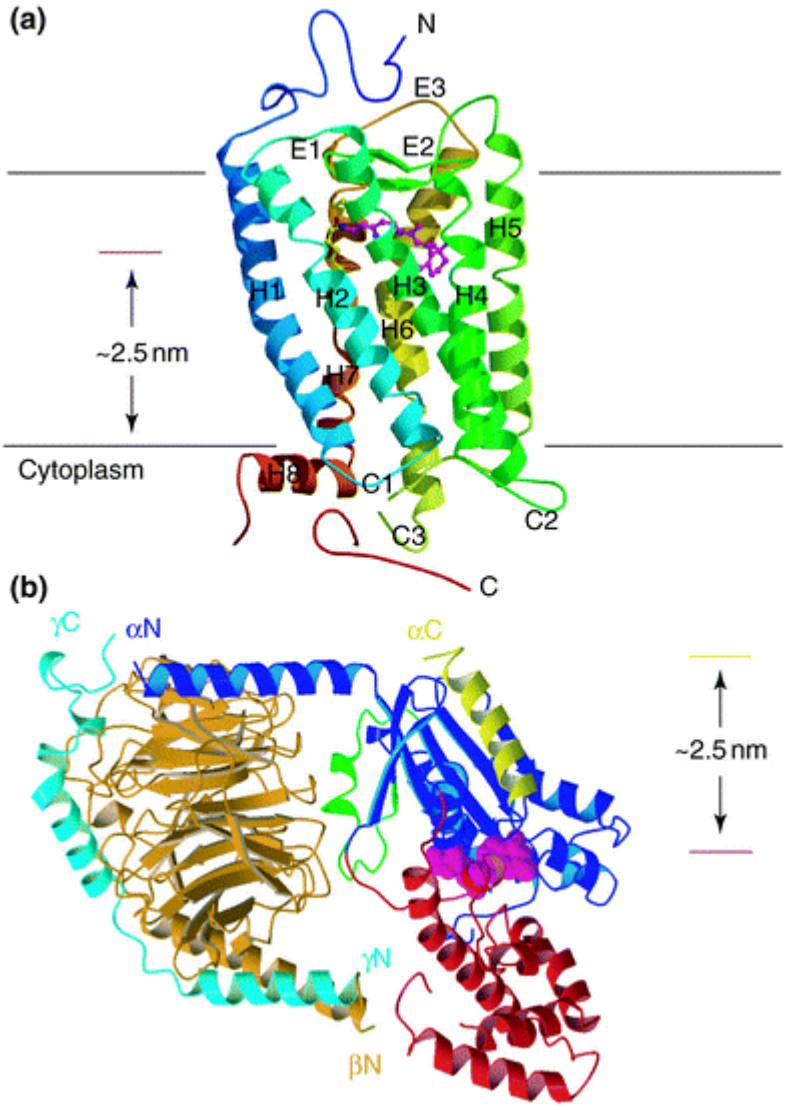

Fig. 5 Interactions of Rhodopsin with Transducin at the Cytoplasmic Surface of the Rod Outer Segment Disc Membrane (From Sakmar, 2002)

(a) Ribbon diagram of rhodopsin prepared from the crystal structure coordinates of the A chain (PDB 1F88). The amino terminus (N) and extracellular surface are shown toward the top of the figure and the carboxyl terminus (C) and intracellular surface toward the bottom. Seven transmembrane segments (H1–H7), characteristic of GPCRs, are shown. The retinylidene chromophore (magenta) is shown in a ball-and-stick format. The crystal structure does not resolve a small segment of the C3 loop linking H5 and H6 or a longer segment of the carboxy-terminal tail distal to H8. Asparagine-linked carbohydrates and palmitoyl groups revealed in the structure are not shown for clarity. The Schiff base linkage of the chromophore lies approximately 2.5 nm from the cytoplasmic surface of the membrane bilayer.

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(b) The GDP-bound form of transducin (PDB 1GOT), with the surface that presumably interacts with rhodopsin facing upward. The Ras-like domain of the α subunit (blue) is seen above the GDP-binding pocket and the helical domain below (red). The α5 helix is shown in yellow and the Switch II region in green. The β subunit (gold) and γ subunit (turquoise) are to the left. The bound GDP (magenta) may be up to 2.5 nm from the surface of transducin. The amino and carboxyl termini of each subunit are labeled. Structures thought to interact with rhodopsin or the membrane, or both -- including the amino and carboxyl termini of the α subunit and the carboxyl terminus of the γ subunit -- cluster on a common surface of transducin. The relative orientation of the cytoplasmic surface of rhodopsin and the rhodopsin-binding surface of transducin is arbitrary. Upon formation of the rhodopsin–transducin complex, the chromophore-binding pocket of rhodopsin becomes allosterically coupled to the nucleotide-binding pocket of transducin, which is approximately 5.0 nm away. The structure of the active state of rhodopsin (R*), or of the R*–transducin complex, has not yet been determined.

In both Gt and Gi heterotrimeric crystal structures, the amino-termini of Gá subunits are resolved as á-helices interacting with Gâã (Lambright et al., 1996; Wall et al., 1995). Several key observations characterize the process of R*-catalyzed nucleotide exchange. In the absence of a catalyst, the rate-limiting step in nucleotide exchange is release of GDP from Gtα to form empty-pocket Gt, Gtα(e)βγ. Gtα(e) is by itself very unstable. R* catalyzes nucleotide exchange by inducing GDP release and stabilizing the reaction intermediate, Gtα(e)βγ. The empty-pocket Gt can be dissociated from R* by either GDP or GTP; R* and nucleotide binding are mutually exclusive. GTP binding is nearly irreversible since conformational changes in the Switch II region destroy the Gtβγ -binding site and induce dissociation of Gtα(GTP) from Gtβγ and R*. R* interacts specifically with heterotrimeric Gt; Gtβγ appears to be absolutely required for efficient R*-catalyzed nucleotide exchange on Gtα (Fung et al., 1983). It is unclear whether Gtβγ plays a mechanistic role in catalysis (Iiri et al., 1998) or whether it merely facilitates binding between Rho and Gtα (Phillips et al., 1992). Binding of Gt to R* and dissociation of GDP appear to be distinct steps; Rho mutants have been described that bind Gt but do not induce GDP release (Ernst et al., 1995 and Fanke et al., 1990).

The molecular mechanism by which R* induces GDP release from Gt is the least-understood step in the Gt signaling cycle. Despite a great deal of data regarding structures of Gt and Rho that interact with each other, little is known about the detailed structure of the complex (Liu et al., 1995). The structure of R* is not known, and the conformational

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changes, that occur in R* and Gt-GDP upon complex formation are not known. Few sites of point-to-point contacts between R* and G protein have been reliably identified (Acharya et al., 1997), and Yoshiki et al (2001) described that both the N and the C termini of Gtα are in close vicinity to the third cytoplasmic loop of rhodopsin in the complex between rhodopsin and transducin. Crystallographic analysis of the R*-Gt(e) complex may prove difficult owing to the instability of R*. The alignment of the interacting surfaces in the structures of Rho and Gt produces a hypothetical low-resolution model of the complex (Bourne et al., 1997) (Fig. 4). These analyses, although lacking details, do suggest clearly that the cytoplasmic loops of Rho, which are roughly 1.5 nm long (at most) are too short to contact directly the nucleotide-binding pocket of Gtα, which is at least 2.5 nm from the Rho-binding surface of Gt. Consequently, R* must act "at-a-distance" to induce nucleotide exchange in Gtα (Iiri et al., 1998).

1.3 Assays of Photoreceptor Activity

1.3.1 UV/Vis Spectra to Study Photoreaction of Rhodopsin

Binding of the prosthetic group, the 11-*cis*-retinal, chromophore to the apoprotein yields rhodopsin, which has a broad absorption band in the visual region of the spectrum with a peak at 500 nm. The different rhodopsin intermediates can be monitored by their different absorption spectra. Meta I (478 nm) and Meta III (470 nm) have absorption maxima significantly different from the tautomeric form interacting with transducin, meta II (380). Transducin binding to Meta II stabilizes this form. Thus, if the conditions (pH, temperature, hydrophobic environment of rhodopsin) are such that the meta I $\Leftrightarrow$ meta II equilibrium favors the former state, transducin binding leads to the so-called “extra-Meta II” signal, an enhanced absorbance at 380nm. The change of this equilibrium can be measured by rhodopsin UV/Vis spectra (see e.g. Ernst et al., 2000; Meyer et al., 2000).

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1.3.2. 1 Trp Fluorescence to Study Retinal-Rhodopsin Interaction

It has been proposed earlier that the five Trp residues located in the transmembrane helices of rhodopsin are able to transfer fluorescence energy to the retinal. Consistently, the decay of rhodopsin to opsin and retinal which follows its activation was found to be accompanied by an increase in the Trp fluorescence (Farrens et al., 1995). This allows to directly monitor the decay. Trp fluorescence of rhodopsin can also be used to study the kinetics of 11-cis-retinal binding to opsin (Reeves et al., 1999).

1.3.2.2 Trp Fluorescence to Measure GTP Binding by Transducin

Trp-207 of Gtα, which is located in switch helix 2, close to the nucleotide binding site (Remmers et al., 1996), exhibits a nucleotide-dependent fluorescence which can be employed to detect the uptake of GTP, GTPγS or AlF^{4-}, which all activate transducin (Philips et al., 1988). This fluorescence monitor is commonly used to study Gt activation catalyzed by rhodopsin (Ernst et al., 2000).

1.3.3 NIR Light Scattering Due to Transducin-membrane Binding

Binding (or dissociation) of transducin to (or from) vesicles or ROS disc membranes lead to changes of near-infrared (NIR) light scattering, measured as changes in transmission (Hofmann, 1992; Heck et al., 2000). Rhodopsin activation leads to complex of membrane-bound transducin with R*, thus, membrane space is liberated which can be occupied by soluble Gt. It has been shown that the light scattering changes are stoichiometric to the formation and dissociation of transducin/R* complexes (Kuhn et al., 1981). NIR light scattering was used to determine the activation energy of Gt binding to the disc membrane (Schleicher et al., 1987). The dissociation of transducin from the disc membrane upon activation by GTP uptake can be followed as decrease in the light scattering intensity.

If NIR light scattering is observed in an analogous system (washed disc membranes plus transducin and GTP) which is supplemented with PDE, a fast increase of the scattering

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intensity is observed upon rhodopsin activation. This signal has been related to the binding of Gtαβγ•GTP to membrane-bound PDE, thus it allows the direct observation of effect activation in the transduction cascade (Heck et al., 1993).

1.3.4 Evanescent Wave Techniques

Evanescent wave techniques allow to follow processes at interfaces between sensor surface and the bulk solution. Using appropriate reconstitution techniques to mimic a biomembrane, the rhodopsin-catalyzed solubilization of transducin in presence of GTP can be followed by these techniques (Ernst et al., 2000 and Heyse et al., 1998).

1.3.4.1 Resonant Mirror Spectroscopy

The resonance mirror technique (Pockrand et al., 1977), as used in a commercially available instrument (IAsys, Affinity Sensors, Cambridge, United Kingdom), makes use of a thin monomode waveguid that is highly sensitive to changes in the surface refractive index. These changes at the sensor surface, due to adsorption or desorption of proteins from/to the aqueous environment, are expressed in the changes of "resonance" angle of incident light at which waveguiding and propagation of the light along the surface of the device are maximal. In practice, 670-nm light from a laser diode is coupled via prism coupling into the waveguide, which forms the bottom surface of a stirred 200-µl cuvette, and reflected light from the inside of the resonant mirror is measured as a function of incident angle. In a resonant mirror sensorgram, the angle where the light propagates maximally is plotted versus time.

The drawback of some commercial sensoring devices is that information about the chemical specification of the sensor chip is limited and that control measurements often have to be obtained from individually prepared samples. However, an approach like the surface plasmon resonance (SPR) method described later, allows identification and correction of nonspecific interactions, which in some cases contribute a major part of the signal.

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1.3.4.2 Surface Plasmon Resonance (SPR)

The physical principles of SPR and its applications for biomolecular interaction analysis have been extensively described elsewhere (Malmqvist, 1993; Malmqvist and Karlsson, 1997; Myszka, 1997; Morton et al., 1998; Fivash et al., 1998; Schuck, 1997). Briefly, one of the interacting molecules (ligand) is immobilized on the sensor surface in a detector, and the other molecule (analyte) is delivered in solution. In the most commonly used commercial SPR detector, the BIAcore instrument, the ligand is attached to a sensor chip consisting of a thin (50 Å) layer of gold on a glass slide (See 2.2.10). The analyte solution is perfused through a microflow cell (volume <0.1 µl) created on the chip. Binding of an analyte to the ligand elevates its local concentration at the surface, gradually increasing the refractive index of the medium close to the gold layer. This has an effect upon the interaction between free electrons (plasmons) in the metal and photons emitted by the instrument's 760 nm light source. Since these changes are proportional to the mass and number of molecules at the sensor surface, the rate of ligand–analyte binding is recorded in real time, which permits measurements of the apparent association and dissociation rates. Following the introduction of the BIAcore instrument in 1991, the SPR method has been widely used in many areas; however, relatively little work has been done on G proteins. The main impediment is the availability and relative instability of the proteins involved in the cascade in detergent solution.

The first success in applying SPR biosensors to the study of the G protein cascade was achieved with stable molecules. For instance, the 10 kDa ã subunit of a G protein effector enzyme cGMP phosphodiesterase (PDEã) could be covalently coupled using the protocols recommended by the BIAcore manufacturer (Slepak et al., 1995). Binding kinetics with the GTPãS-bound form of the Gt protein transducin and áâ subunits of PDE was measured, and several PDEã mutants were screened.

The most interesting application of the SPR method for analyzing the G protein cascade is to study protein–protein interactions in a natural setting, that is, at the surface of a phospholipid membrane. The kinetic constants obtained in such an environment will mimic the real binding parameters better than those obtained by traditional SPR applications or other optical methods using buffer solutions with detergents. Analysis of G protein-

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coupled receptors (GPCRs) is particularly important because this method could be applied for screening of drugs. It is important to note that more than 50% of currently used pharmaceuticals act on GPCRs. Because of the availability of rhodopsin, the most abundant of all GPCRs, to date, most evanescent field experiments have been done on the rhodopsin–transducin system, which serves as a model to show the feasibility of immobilization the GPCRs on solid supports. To investigate rhodopsin in a lipid layer, Salamon et al. (1996) used an in-house SPR spectrometer (not the commercial BIAcore), where the sensor surface consisted of a silver film deposited on a glass prism. After a phosphatidylcholine bilayer was created on the surface by direct adsorption of lipids on silver metal, rhodopsin was incorporated by simply adding octyl-glucoside-solubilized bovine photoreceptor outer segment membranes so that the detergent was diluted below the critical micelle concentration. Using this experimental design, the researchers observed distinct changes of SPR spectra at each of the following steps: (1) adsorption of the lipid on the bare silver layer; (2) incorporation of rhodopsin; and (3) addition of transducin. It is interesting that the SPR spectra changed reversibly upon irradiation of rhodopsin by yellow light, indicating that a change not only in overall mass, but also in conformation could be detected.

Another successful application of the SPR was performed by Heyse et al. (1998), who also used a noncommercial SPR spectrometer with mercaptan-derivatized gold sensor surface.

Bieri et al (1999) used carbohydrate-specific chemistry for the oriented and patterned functional immobilization of a representative GPCR. The reconstituted receptor–G protein system was remarkably table. By surface plasmon resonance (SPR), it was able to follow ligand binding, G protein activation, and receptor deactivation of a representative GPCR, bovine rhodopsin. Microcontact printing was used to produce micrometer-sized patterns with high contrast in receptor activity.

Along with phospholipid, the membranes contained a synthetic thiolipid with a spacer arm attached to the phosphate moiety. The arm protruding from the plane of the polar lipid heads secured the space between the membrane and the gold. Another feature of the developed experimental setup was photo-bleaching of the modified surface, which created micrometer-sized alternating regions where reactive groups on the gold were interrupted

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by inactivated areas. This produced a pattern containing areas of the thiolipid covalently attached to the gold and areas of pure phospholipid membrane separated from the surface by the water phase. As one would expect, rhodopsin was incorporated preferentially into the phospholipid bilayer regions, apparently because the space could accommodate the extracellular loops, whereas transducin was uniformly distributed over the entire outer surface facing the mobile phase. Quantitative parameters of rhodopsin-transducin binding closely resembled characteristics of the native system, indicating that the functionality of rhodopsin was preserved. This method appears to be promising for the analysis of the lateral diffusion of the G protein, and it can be extended to receptor-binding proteins such as arrestin, G protein coupled receptor kinases (GRKs), and perhaps other proteins in the cascade.

1.4 The Enzymatic Biotinylation of Proteins

The rapid expansion of technologies based on the interactions of biotin with its very-high-affinity binding proteins, avidin and streptavidin, in biochemistry, immunology, cell biology and biotechnology, might obscure the fact that biotin is, in the first place, a vitamin, required by all forms of life. It is synthesized by plants, most bacteria and some fungi, and plays vital metabolic roles, but only when covalently bound to protein. Biotin is covalently attached at the active site of certain enzymes that transfer carbon dioxide from bicarbonate to organic acids to form cellular metabolites. These biotin-dependent enzymes have key roles in gluconeogenesis, lipogenesis, amino acid metabolism and energy transduction. Most organisms have fewer than five protein species that are biotinylated and thus, the reaction that attaches biotin to these proteins is remarkably specific. In addition, in some bacteria, the enzyme that catalyzes biotin attachment also represses transcription of the genes involved in biotin biosynthesis.

1.4.1 The Biotinylation Reaction

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Biotin protein ligase (BPL) is the enzyme responsible for attaching biotin to a specific lysine at the active site of newly synthesized biotin carrying enzymes. This is a post-translational modification of extraordinary specificity. In *Escherichia coli*, only a single lysine residue within the biotin carboxyl carrier protein (BCCP) subunit of acetyl-CoA carboxylase is recognized and biotinylated by BirA (the BPL of *E. coli*). In most other organisms up to five different proteins are biotinylated (Cronan, 1990). These include four key metabolic enzymes – acetyl-CoA carboxylase, propionyl-CoA carboxylase, pyruvate carboxylase and methylcrotonyl-CoA carboxylase and other enzymes such as transcarboxylase and oxaloacetate decarboxylase, which are involved in specialized metabolic transformations (Samols, 1988). This tightly restricted specificity of biotinylation results from the recognition of a complex protein domain by biotin holoenzyme synthetase. These biotinylated domains are highly conserved in a wide variety of species, and reside in an eighty amino acid region surrounding the modified lysine (Samols et al., 1988). A related 13-aa consensus sequence defines the minimal substrate for this enzyme in vivo (Schatz, 1993). Tagging a recombinant protein at either end with such a peptide will cause it to be biotinylated in *E. coli*. The biotin moiety can be used as an affinity tag to purify the protein on monomeric avidin resin (Kohanski and Lane, 1990).

Biotin attachment is a two-step reaction that results in the formation of an amide linkage between the carboxyl group of biotin and the ε-amino group of the modified lysine (Fig. 6) (McAllister and Coon, 1966). The BPL reaction is closely analogous to that of the amino acyl-tRNA synthetases. The functional interaction between BPLs and biotin enzymes is highly conserved throughout nature, as biotinylation will occur when the two proteins, BPL and the biotin enzyme, are derive from such divergent species as bacteria and humans, (McAllister and Coon, 1966; Leon-Del-Rio et al., 1995; Tissot et al., 1998). Moreover, the BirA proteins of *E. coli* and *Bacillus subtilis* also act as transcriptional repressors that regulate the biosynthesis of biotin (Cronan, 1989).

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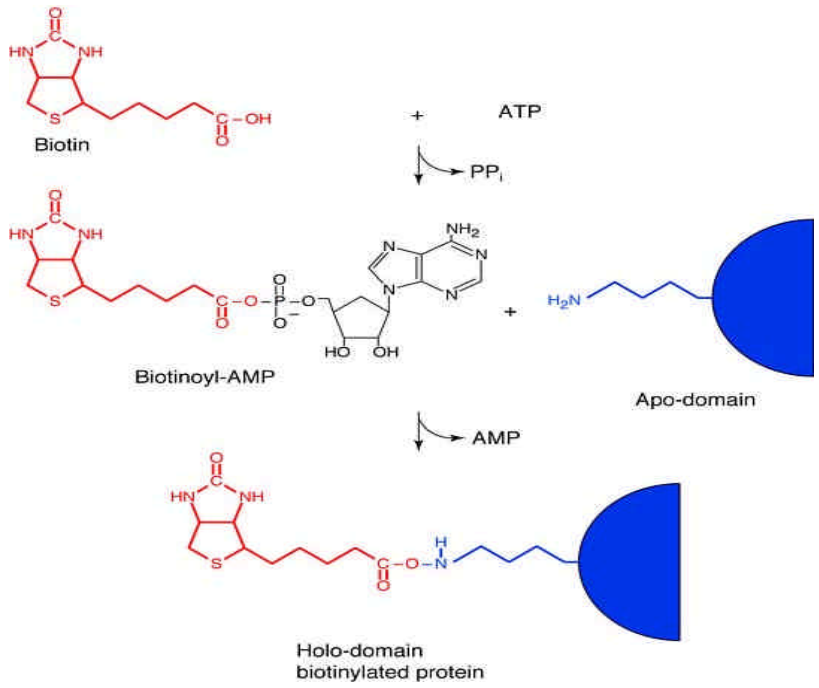

Fig. 6 The Biotin Protein Ligase (BPL) Reaction

1. 4. 2 Uses of Enzymatic Protein Biotinylation

Given recombinant DNA technology, it is a simple matter to produce chimeric proteins in which a biotin accepting domain has been fused to a protein of interest (Cronan, 1990). Such fusion proteins become efficiently biotinylated in vitro and in vivo. The biotin domain can be located at the native C-terminal location, at the amino terminus, or within the fusion partner protein (an in-frame fusion) (Cronan, 1990; Consler, et al., 1993). In each case it seems that a linker or hinge region such as those often found upstream of the bacterial biotin domains should be included to allow independent folding of the domain. These attributes together with avidin: streptavidin technology allow biotinylation to be a powerful tool. Fusion proteins can be readily purified in the denatured state on columns of immobilized avidin or streptavidin, but can also be purified in native state on columns of low affinity forms of avidin or streptavidin produced by subunit dissociation, amino acid substitutions, or chemical modification (Cronan, 1990). By use of these low affinity columns the fusion proteins can be eluted by addition of biotin to the elution buffer. This

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purification tag has the advantage that it can be used for proteins produced in any cell type whereas other tags can be compromised by cell type (e.g. polyhistidine tags work poorly in yeast due to abundant proteins that bind to the metal chelate column). The high affinity columns provide the means to stably tether the fusion protein by a defined interaction rather than at random (Tatsumi et al., 1996). The immobilized fusion protein can then be used as a ligand to isolate other molecules (e.g. antibodies). In vivo biotinylation has also been used as a sensitive reporter of protein sorting in both procaryotes and eucaryotes (biotin domains seem able to readily transverse biological membranes) (Reed et al., 1991; Ackerman et al., 1992; Jander et al., 1996). Moreover, the single biotin born by each protein can be used to tether it to avidin or streptavidin coated surfaces for various purposes, such as the construction of a protein affinity column, the development of a scintillation proximity assay for drug screening, for ligand binding experiments that utilize surface plasmon resonance technology (Bieri et al., 1998; Heyse et al., 1998). The chief advantage of this approach is that, unlike chemical reagents, enzymatic biotinylation assure that all molecules will be immobilized in a uniform orientation.

1.5 Motivation and Goal of this Project

The goal of this project is to explore a new approach to introduce a biotin moiety at the N-terminus of rhodopsin. The aim is to express recombinant rhodopsin with a biotin-tag which is inserted at the N-terminus. This should allow oriented immobilization of rhodopsin at the sensor surface and reconstitution into a lipid bilayer. Finally, activation of Gt by the immobilized rhodopsin will be monitored by the IAsys system and will lead in the ideal case to a routine technique for assaying interactions of GPCR-G protein on a molecular level.

For the proposed subject, I want to add a suitable sequence of 13 amino acids (Schartz, 1993), a so called biotin-tag, to the N-terminus of rhodopsin. Enzymatic biotinylation will be used to get site-specific biotinylated rhodopsin for immobilization in an uniform orientation to the sensor chip of the resonant mirror (IAsys system).

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