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WHAT SITE-DIRECTED LABELING STUDIES TELL US ABOUT THE MECHANISM OF RHODOPSIN ACTIVATION AND G-PROTEIN BINDING

David L. Farrens

Department of Biochemistry and Molecular Biology, School of Medicine, Oregon Health & Science University, Portland, Oregon 97239

Abstract

Rhodopsin is the photoreceptor protein responsible for dim-light vision in mammals. Due to extensive biophysical, structural and computational analysis of this membrane protein, it is presently the best understood G-protein coupled receptor (GPCR). Here I briefly review one approach that has been extensively used to identify dynamic and structural changes in rhodopsin - the use of site-directed labeling methods (SDL) coupled with electron paramagnetic resonance (EPR) and fluorescence spectroscopy. These SDL studies involve introducing individual cysteine residues into the receptor, then labeling them with cysteine-reactive probes for subsequent analysis by the appropriate spectroscopy. I will give a brief overview of how SDL methods are carried out and how the data is analyzed. Then, I will discuss how SDL studies were carried out on rhodopsin, and how they were used to identify a key structural change that occurs in rhodopsin upon activation -movement of transmembrane helix 6 (TM6). I will also briefly discuss how the SDL studies of rhodopsin compare with SDL studies of other GPCRs, and compare the SDL data with early and recent crystal structures of rhodopsin. Finally, I will discuss why the TM6 movement is required for rhodopsin to couple with the G-protein transducin, and speculate how this mechanism might be a universal method used by all GPCRs to bind G-proteins and perhaps other proteins involved in visual signal transduction, such as arrestin and rhodopsin kinase.

INTRODUCTION

Light triggers rhodopsin and the visual signal transduction

The basic biochemistry of signal transduction in the retina has been known for some time. Light activates the photoreceptor membrane protein rhodopsin by converting its 11-*cis* retinal chromophore to all-*trans* retinal. The 380 nm absorbing species thus produced, Meta II, activates a number of G-proteins (transducins), inducing them to take up GTP, and then stimulate a cGMP phosphodiesterase to hydrolyze cGMP. The end result is closure of cation conductance channels and the generation of a nerve signal.

However, the above description does not address two fundamental mechanistic questions – *how* does the structure of rhodopsin change upon “activation”, and *why* does this change make it able to suddenly bind and activate G-proteins? These questions have been the focus of intense study over the years, due to their central importance for understanding vision at a molecular level, and because rhodopsin has emerged as a key model for understanding how the large family of G-protein coupled receptors (GPCRs) work.

In this review, I discuss one type of approach that has proven especially fruitful for studying these types of questions: site-directed labeling (SDL), coupled with electron paramagnetic resonance (EPR) or fluorescence spectroscopy. First, I will give a brief overview of how these SDL approaches are carried out, and then discuss how SDL approaches have identified

key structural changes that occur in rhodopsin upon light-activation, and then review why these structural changes are thought to convert rhodopsin into an active, signaling receptor that can bind a G-protein. I will also discuss how the SDL data on rhodopsin compare with different crystal structures of rhodopsin and with SDL studies that have been carried out on other GPCRs.

OVERVIEW OF SITE-DIRECTED LABELING (SDL) METHODS

Why and how SDL studies are performed

SDL approaches are employed for difficult to study biomolecules (such as membrane proteins like rhodopsin), because they enable the study of dynamic protein regions in real time, and they require only minimal amounts of sample (important factors when carrying out studies of some membrane proteins, like rhodopsin, that require expression in eukaryotic systems).

In brief, the first steps in an SDL study usually involve introducing, through mutagenesis, individual cysteine residues into the region of interest in the protein. A “background” protein sequence which contains no reactive cysteines is used. To obtain insights into the structure around the site, the cysteine is first reacted with a sulfhydryl-reactive spectroscopic reporter group and then studied using the appropriate method (i.e., a fluorescent label is used for fluorescence studies, and a spin-label for EPR studies). Although structural insights about the site of attachment can also be gained from determining the chemical reactivity of the introduced cysteine (that is, how readily it is labeled), this approach will not be discussed extensively here.

How SDL data are measured and interpreted

In order to interpret SDL data in a structural context, a number of aspects are assessed. These factors include the mobility of the attached probe, the exposure of the probe to solvent, and the relative distance of the probe to other sites on the protein. The SDL approach is shown in a cartoon format in **Figure 1**, and the way SDL data are collected and analyzed is discussed in more detail below.

Mobility measurements—The mobility (or rotational correlation time) of a probe reflects steric constraints imposed by the surrounding protein structure. Thus, this value directly reflects on the structure surrounding the site of label attachment. Decreased mobility indicates a probe is sterically constrained (due to interaction with other parts of the protein), rather than being located on the “outside” of the protein.

In EPR SDL studies, the mobility of the spin-label probe can be qualitatively assessed by comparing the EPR lineshapes ¹, and more quantitatively, by comparing the peak-to-peak width of the central MI = 0 line ². For fluorescence SDL studies, the fluorescence anisotropy of the probe is measured to determine the mobility of the probe. A higher anisotropy indicates the probe is less able to lose the polarization of the initial exciting light and thus is more constrained/less mobile. Anisotropy can be assessed using steady state fluorescence measurements, and even more information can be gained by carrying out time-resolved fluorescence anisotropy measurements ³⁻⁴.

Solvent accessibility—The accessibility of the probe to solvent (and external agents dissolved in the solvent) is used in SDL studies to delineate local structure and identify sites of conformational change. In EPR SDL studies, collision of a spin label with paramagnetic agents (such as O₂ and Ni:EDDA) causes a decrease in the T₁ of the spin label. This then broadens the signal and appears as a “quenching” of the intensity of the EPR spectra.

Because the Ni:EDDA has a preferential solubility in the aqueous domain, and the O₂ more so in the membrane, a combination of both agents can be used to determine the solvent accessibility (and thus environment) of the spin-label on each of the mutant samples, using a procedure called power saturation measurements⁵⁻⁶.

For fluorescence studies, the accessibility of the probe is assessed by determining how often external quenching agents collide with and quench (decrease) the probe's fluorescence. For the study of membrane proteins, both aqueous quenchers (such as KI) and hydrophobic quenchers (such as spin-labeled lipids) can be employed. The results are then used to define the relative orientation of the probe on the surface of the protein⁷ or relative to the membrane. For some probes, the wavelength of maximum fluorescence emission (emission maximum) can also be used to infer solvent accessibility of a site. For example, the emission maximum of the fluorescent probe bimane is blue-shifted when in a solvent of lower polarity, such as occurs when a site is inaccessible to water. Such shifts have been shown to reliably report on the apparent polarity and solvent accessibility of the site on the protein to which it is attached⁸.

Distance measurements—A challenging yet informative type of SDL study involves measuring distances between sites in the protein, as this enables the mapping of local structure, and defining the direction and type of movements that occur for example upon receptor activation.

In EPR SDL studies, distances between pairs of sites are determined by measuring the amount of dipole-dipole coupling between pairs of spin-labels in double cysteine mutants. The proximity between the probes is assessed by measuring the amount of peak broadening that this interaction induces in the EPR spectra⁹⁻¹². More recently pulsed EPR methods are being used, as these significantly enhance the precision of distance measurements¹³⁻¹⁶.

For fluorescence SDL studies, distances have traditionally been measured using fluorescence resonance energy transfer (FRET) based methods. However, FRET studies of rhodopsin are complicated by the significant FRET that occurs from the attached probe to the retinal chromophore. Furthermore, FRET occurs over distances too large to precisely define the type of subtle, small conformational changes that are often involved in converting a protein structures into an active state.

To overcome some of these problems with FRET, our lab has been developing a new simple fluorescence SDL method for studying proteins. The method exploits the fact that a Trp residue can significantly quench the fluorescence from some fluorescent probes¹⁷⁻¹⁸, and we have established that the amount of this quenching is related to distance between the probe and the Trp residue. We now refer to this approach TrIQ (for Trp-Induced Quenching).

In order to carry out a TrIQ study, one first introduces a unique cysteine near a Trp residue (the Trp can either be a native residue or be introduced through mutagenesis). The cysteine is then labeled with a TrIQ-sensitive fluorophore. The relative proximity between this Trp-fluorophore pair is then assessed by comparing it to a control sample (in which the Trp residue has been substituted with a non-quenching Phe residue). Increased TrIQ (less fluorescence) indicates close proximity, and changes in intensity upon protein activation indicate a conformational change occurs at that site.

The TrIQ method has a number of unique advantages, especially for studies of rhodopsin and other GPCRs. TrIQ is ideal for assessing short-range distances and small changes in protein structure, because it is only sensitive over short distances (substantial TrIQ only

occurs when the Trp and probe are at or near contact with each other). Only one cysteine needs to be labeled, and samples with less than 100% labeling efficiency can still be studied (because TrIQ monitors the fluorescence of the probe, not the Trp, and the Trp is always present in the protein sequence). Finally, TrIQ studies require only microgram amounts of sample and need only relatively simple and widely available instrumentation. For these reasons, TrIQ complements FRET and spin-spin interaction studies, and eliminates several technical problems often faced in measuring intra-protein distances and dynamics in proteins.

Until recently, TrIQ studies have employed the fluorophore bimane. However, our lab has recently expanded the TrIQ method to now include several different fluorophores of different sizes and spectral properties¹⁹. For these probes, the distance dependence of TrIQ is directly related to the size of the probe - smaller probes show substantial TrIQ only over relatively short C α -C α distances (~5-10 Angstroms). We determined that an analytical method can also be applied to the analysis of TrIQ data¹⁹. This method makes it possible to quantify exactly how many Trp/fluorophore pairs are in direct contact with each other at the moment of light excitation. Such pairs are non-fluorescent due to what is called “static quenching”. This analysis enables one to obtain a fast (subnanosecond) “snapshot” of how much of the protein has a given conformation with the Trp/probe pair in contact, and to monitor shifts in the dynamic equilibrium between different protein structural states. Examples of information gained from EPR SDL, fluorescence SDL and TrIQ studies on rhodopsin are discussed below.

HELIX 6 IN RHODOPSIN MOVES UPON LIGHT ACTIVATION

The vast majority of SDL studies on rhodopsin have focused on the cytoplasmic face of the receptor. In fact, essentially the entire cytoplasmic face of rhodopsin has been subjected to cysteine mutagenesis and study by SDL methods using the principles described above. The data from these studies have been analyzed to determine the local protein structure and to identify sites of light-induced conformational change in the receptor, and to understand how these changes enable rhodopsin to couple with its signaling partners (such as transducin, rhodopsin kinase and arrestin). The majority of the studies employed EPR SDL spectroscopy (reviewed in²⁰) and cysteine reactivity measurements²¹⁻²⁴. However, a substantial number of sites were also subjected to fluorescence SDL studies²⁵⁻²⁶, and in some cases F19 NMR studies²⁷⁻²⁹.

Overall, these studies showed the most dramatic light-induced changes were detected for sites on or flanking the cytoplasmic end of transmembrane helix 6 (TM6). Taken together, these data are interpreted to indicate that rhodopsin activation primarily involves some kind of tilt and/or rotation of TM6^{25, 27, 30-31}, although it should be noted that probes attached at Helix 8 also detect some changes^{3, 32-35}.

Intriguingly, early results suggested that TM6 movement is necessary for the formation of a receptor that can couple with G-protein. For example, when TM6 was linked with TM3 through disulfides³⁰⁻³¹ or a zinc binding site³⁶, the ability of rhodopsin to activate transducin was severely impaired. In contrast, introducing disulfide cross-links at other sites in the cytoplasmic face of rhodopsin did not kill its ability to activate transducin^{31, 37}, further emphasizing the unique importance of TM6 movement in rhodopsin activation.

SDL studies compare well with Rhodopsin Crystal Structures

How do these SDL studies compare with the crystal structures of rhodopsin? Overall, they are in excellent agreement, although there has been some interesting initial differences. For example, the early EPR SDL studies predicted that TM5 would form an extended helix⁵,

yet this was not observed in the first crystal structure of rhodopsin³⁸. However, subsequent rhodopsin crystal structures do show a more extended TM5 helix, and this intriguing discrepancy might suggest that the loop connected TM5 and TM6 is conformationally flexible and can adopt different structures under different conditions, perhaps indicating this region may have to move during transducin binding and activation³⁹.

Another discrepancy involved comparison of SDL data with the structure of an early photo-activated, de-protonated intermediate of rhodopsin⁴⁰. This structure did not show the same amount of TM6 movement as predicted by the EPR and fluorescence SDL studies, possibly because it represents the MIIa form of rhodopsin, and the SDL data reflect the MIIb form of activated rhodopsin⁴¹. More recently, structures of “active opsin” and active-opsin bound with a fragment of transducin have been solved⁴²⁻⁴³, and these structures do show TM6 undergoing a movement very similar to that predicted by the body of SDL studies. A cartoon Figure of the TM6 movement proposed from EPR SDL studies, and how it compares with crystal structures of rhodopsin and active opsin is given in **Figure 2**. Interestingly, TM6 movement appears to occur during light activation of many other light-sensitive retinal containing membrane proteins, including bacteriorhodopsin⁴⁴⁻⁴⁷, sensory rhodopsin⁴⁸⁻⁵⁴, suggesting the movement is a universal feature for these proteins.

HELIX 6 MOVES TO ENABLE BINDING OF THE C-TERMINUS OF THE TRANSDUCIN G α SUBUNIT

As mentioned above, early SDL and cross-linking data suggested that TM6 movement might be a key step required to enable G-protein binding. Our lab used fluorescence SDL and TrIQ to investigate why this movement might be necessary to form functional receptor²⁶. To do this, we employed peptide analogues of the C-terminal tail of the transducin G α subunit (G α _T). This region of transducin is required for transducin binding⁵⁵, and the G α _T C-terminal peptide analogues are known to bind rhodopsin with high affinity⁵⁶ and become structured upon binding⁵⁷⁻⁵⁸. The results, summarized below, indicated that TM6 movement occurs to expose a necessary hydrophobic binding site for the (G α _T) C-terminal tail.

Our studies were carried out as follows. First, we performed cysteine reactivity and fluorescence SDL quenching studies on a rhodopsin mutant containing a unique cysteine residue in the inner face of TM6 (V250C), in the presence and absence of the G α _T C-terminal tail peptides. These studies indicated the G α _T C-terminus peptide bound and interacted with the inner face of TM6 (the side of the helix that would become exposed upon TM6 movement), consistent with other data suggesting a role for this region of rhodopsin for interacting with the G α _T C-terminal tail⁵⁹⁻⁶⁰.

To further identify and map the binding site for the G α _T C-terminus on rhodopsin, we then prepared rhodopsin mutants containing cysteines at a number of different sites in the cytoplasmic face surrounding TM6, and labeled these mutants with the TrIQ-sensitive fluorophore bimane. We also introduced a Trp residue into the G α _T C-terminal tail peptide, and measured the effect of adding this peptide to the bimane labeled rhodopsin samples. A subset of these sites showed a decrease of bimane fluorescence upon peptide binding (i.e., increased TrIQ), indicating proximity between the Trp-containing part of the peptide and that site on rhodopsin and thus further localizing the G α _T C-terminal tail binding site on rhodopsin.

We then used this TrIQ-based peptide binding assay to identify what physical mechanism was the cause for the high affinity interaction between the transducin G α _T C-terminal tail and rhodopsin, by carrying out these studies on rhodopsin mutations that were deficient in

their ability to activate transducin⁶¹. These TrIQ data revealed that a key role in $G_{T\alpha}$ C-terminal tail binding involves a high affinity interaction with hydrophobic residues in rhodopsin (L226, T229 and V230). Moreover, the results from the TrIQ based peptide binding studies determined that each of these residues contributes ~ 3 kcal/mol binding energy for interaction with the $G_{T\alpha}$ C-terminal tail. Based on these results, we proposed that the reason why TM6 movement occurs in rhodopsin during light activation is to expose the “hydrophobic patch” in rhodopsin (made up of residues L226, T229 and V230), to directly interact with hydrophobic residues in the $G_{T\alpha}$ C-terminal tail of transducin²⁶.

How does this TrIQ study compare with the recent structure of “active opsin” bound to a similar $G_{T\alpha}$ C-terminal peptide⁴³? As shown in **Figure 3**, the SDL and TrIQ results are in excellent agreement with the crystal structure data. The binding site of the $G_{T\alpha}$ C-terminal peptide agrees with the site proposed from the SDL and TrIQ data. Furthermore, the specific hydrophobic residues in the “hydrophobic patch” exposed during the activation of rhodopsin are seen to make extensive hydrophobic interactions with hydrophobic residues on the $G_{T\alpha}$ C-terminus. Thus, it seems clear that one reason TM6 moves in rhodopsin is to make hydrophobic contacts available for the binding of the $G_{T\alpha}$ C-terminal tail.

DOES HELIX 6 MOVE IN OTHER GPCRS?

The results for rhodopsin described above lead to an obvious question – does TM6 move in other receptors, i.e., is TM6 movement a universal feature of GPCR activation? Unfortunately, due to the technical challenges of expressing and working with ligand-binding receptors, no other GPCR has been subjected to the type of extensive, comprehensive SDL EPR and SDL fluorescence studies that have been carried out on rhodopsin. Thus an extensive comparison between data sets for two different GPCRs is not yet possible. However, a substantial number of fluorescence SLD studies have been carried out on the beta-adrenergic receptor (BAR) by the Kobilka lab and co-workers. As reviewed briefly below, the evidence from these studies support the hypothesis that a similar TM6 movement also occurs during the activation of BAR.

Evidence for TM6 movement from early fluorescence SDL studies of the beta-adrenergic receptor (BAR)

Early fluorescence studies on BAR detected movement at TM6, and studies of constitutively active BAR mutants emphasize the importance of this movement for the formation of active receptor⁶²⁻⁶⁴. The nature of these movements was further defined using fluorescence quenching studies. These studies employed spin-labeled lipids to help orient the TH6 helix of BAR and demonstrate conformational changes that were consistent with a TM6 movement⁶⁵. Interestingly, one fluorescence SDL study suggested that the BAR exists in a number of different protein conformational sub-states⁶⁶, based on the presence of multiple fluorescence lifetimes detected in the decay data⁷.

Evidence for similar TM6 movements in rhodopsin and the beta-adrenergic receptor from TrIQ studies

Taken together, the BAR results described above strongly supported the hypothesis that TM6 movement is a conserved and primary step shared in the activation process of GPCRs. Subsequent TrIQ studies provided more direct evidence for TM6 movement and the similarity of this movement in both rhodopsin and the BAR.

To test if a TrIQ SDL study could detect TM6 movement in rhodopsin, Dr. Jay Janz in our lab introduced a Trp residue on TM3 (at site 139) and a TrIQ-sensitive bimane label opposite it, on TM6 (at site 250)⁶⁷. Note – these same sites on rhodopsin (see Fig. 2)

showed substantial spin-spin interactions in EPR SDL studies³⁰. The thought was that if TM6 movement occurs, the bimane on TM6 should come closer together to the Trp on TM3, resulting in a decrease in bimane fluorescence (increased TrIQ). As shown in **Figure 4**, this is exactly what was observed. Due to decreased FRET from the probe to retinal in the MII state (explained in Fig. 4B), the bimane probe on site 250 increased in fluorescence upon rhodopsin photo-conversion to MII (Fig. 4C). In contrast, when a sample with a bimane on site V250 and a Trp opposite on TM3 (at site 139) was converted to MII, the increase in bimane fluorescence was abolished. These results are agreement with the proposed outward movement of TM6. .

A much more comprehensive use of this TrIQ approach was used by the Kobilka lab to study the effect of ligands on TM6 movement in BAR. In these studies, they introduced into the BAR a Trp at a site in TM3 and a bimane at TM6 at what are the equivalent sites described above for rhodopsin. They then measured the extent of TrIQ to assess if similar TM 6 movement occurs in BAR as in rhodopsin, and to test if there was a direct correlation of TM6 movement and the binding of different BAR ligands.

The BAR study showed very similar amount of activation-induced TrIQ as was observed in the rhodopsin study discussed above and in Figure 4. A substantial increase in TrIQ (decrease in bimane fluorescence) was observed for the Trp/bimane labeled BAR mutant, but only upon binding agonists (activating ligands) - no change in TrIQ (movement) was observed upon binding of antagonist to BAR⁶⁸. Most importantly, this study showed that the change in TrIQ/TM6 movement directly correlated with the ligand binding affinity.

The BAR study also made a very important observation overlooked in the previous work. The sites on TM3 and TM6 are directly opposite each other in both receptors, and they are physically separated by a salt-bridge between TM3 and TM6 (R135 and E247 in rhodopsin). This salt-bridge is highly conserved in GPCRs, and is thought to keep GPCRs in an inactive state. For substantial TrIQ to occur in these studies, the salt-bridge must break to give the Trp and bimane greater access to each other. Since substantial TrIQ occurred for both the rhodopsin and BAR samples, the data indicate activation of both receptors involves a similar movement and breaking of the salt bridge between TM3 and TM6, providing further compelling support for the hypothesis that TM 6 movement is a shared step in the activation of GPCRs.

The magnitude TM6 movement may control how efficiently a GPCR can activate G-proteins

Recently, fluorescence SDL and TrIQ studies were carried out in order to compare TM6 movements between bovine rhodopsin and lamprey parapinopsin (the latter is a UV-sensitive non-visual pigment found in the pineal organ). These studies were carried out because parapinopsin does not hydrolyze the retinal Schiff-base linkage as part of a photocycle, and interestingly, it is ~ 20X less effective at activating G-protein than rhodopsin. The Terakita lab proposed an interesting hypothesis - the difference in activity between rhodopsin and parapinopsin might be due to differences in TM6 movement. To test this possibility, they carried out SDL and TrIQ studies to compare and contrast movement in these two receptors. Their data suggested the magnitude of TM6 movement in parapinopsin was substantially less than that observed in bovine rhodopsin, consistent with the hypothesis that the large TM6 movement explains the greater efficiency of rhodopsin for activating G protein. This intriguing result suggests that the magnitude of TM6 movement correlates with the ability of a receptor to activate G-protein⁵⁴. This study is discussed in more detail in an accompanying manuscript by Tsukamoto and Terakita.

CONCLUSION AND FUTURE DIRECTIONS

It is tempting to speculate that TM6 movement is a universal activation event in all retinal proteins and GPCRs. Moreover, as discussed above, it is possible that the amplitude of TM6 movement may be a key factor responsible for the wide functional diversity observed among GPCRs. For GPCRs, one can hypothesize that TM6 movement must occur to expose the “hydrophobic patch” to enable high affinity binding of the G-protein, and the frequency or magnitude of this exposure is key to increase the number of binding events. Thus, future drug design should assess the effect on TM6 movements and its role in issues of affinity vs. efficacy. Future crystal structures of constitutively active and retinal-bound rhodopsin, and other GPCRs such as the BAR bound to different agonists will help explain if this is indeed a possibility. The above discussion also leads to the next exciting set of questions - what other role might TM6 movement play besides opening a binding site for G-proteins? For example, is TM6 movement and exposure of the “hydrophobic patch” necessary/required for binding of arrestin or rhodopsin kinase?

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Figure 1. Cartoon diagram showing the different types of information obtained from Site-Directed Labeling (SDL) studies of rhodopsin

Left) Example showing how electron-paramagnetic resonance (EPR) SDL studies are carried out. Spin-labels are attached to the sulfur of cysteine residues introduced into the protein by mutagenesis. Then, depending on the experiment, the labeled samples are studied to determine the accessibility of the probe, its mobility or its proximity to another spin-label. Right) In a similar way, fluorescence SDL studies of rhodopsin are carried out by attaching fluorescent probes to cysteine residues and measuring similar parameters as for the EPR studies. The fluorescent probe indicated in the figure is a bimane label. Further details about how the data is collected and analyzed for the different types of information are discussed in the text.

Dark

Activated

Figure 2. View of cytoplasmic face of rhodopsin comparing TM Helix 6 movement proposed from early SDL studies with recent crystal structure of active opsin

Left half) Early cartoon model of light-induced TM6 movement in rhodopsin derived from EPR SDL studies³⁰. The model shows how a outward TM6 movement was determined by measuring the distance between a spin-label on TM3 (at site 139) and a second spin-label probe placed at different sites opposite on TM6. The dark-state model (top) was constructed by placing the distances measured in the dark on a rhodopsin model based on an early helix packing scheme of Schertler, Unger and co-workers⁶⁹. Then the pattern of distances and distance changes upon light-activation were used to model the relative movement of TM6 in the activated state (bottom). Subsequent results indicating TM6 movement were also obtained from chemical reactivity and fluorescence studies. Right half) models based on the crystal structure coordinates from dark-state rhodopsin (top, PDB 1GZM), and the “active opsin” structure (bottom, PDB 3CAP). In both figures, the colored balls indicate the beta carbons of site 139 (black), site 250 (red) and site 251 (orange). Helix 8 is also indicated. Note the similar relative change in distance between these sites in both the EPR SDL studies and crystallographic structures.

Figure 3. Location and mechanism of interaction between rhodopsin and the transducin $G_{T\alpha}$ C-terminus as determined from site-directed labeling, TrIQ and crystallography studies

A) fluorescence SDL and TrIQ studies determined the location of the $G_{T\alpha}$ C-terminal binding site was at the inner face of TM5 and TM6 in photoactivated rhodopsin²⁶. The study also discovered that a key aspect of this binding involved direct contact between a “hydrophobic patch” on rhodopsin (made up of residues L226, T229 and V230) and the transducin G α C-terminus. B) The subsequent crystal structure of $G_{T\alpha}$ C-terminus peptide bound (yellow) to activate opsin (red) shows the peptide does indeed bind to the predicted binding site. C) Close-up view showing the direct interaction of the “hydrophobic patch” residues on rhodopsin L226, T229 and V230 (red lettering) with key hydrophobic residues on the $G_{T\alpha}$ C-terminal peptide, I340, L344 and L349 (orange lettering). Figure 3A was adapted from Janz and Farrens, 2004²⁶. The original model was made using the coordinates from rhodopsin crystal structure 1U19, and tilting and rotating TM6 outwards as predicted from the studies described in Figure 2. The model in Figures B) and C) were generated using PDB coordinates 3DQB⁴³.

Figure 4. TM6 Movements in rhodopsin detected using TrIQ (Trp-induced quenching) of bimane fluorescence

A) Three-dimensional model of rhodopsin viewed toward the cytoplasmic face. The bimane-label on TM6 (site 250) is shown in purple. The tryptophan quencher introduced opposite of it on TM3 (at site 139) is shown in light green. The large arrow indicates the proposed TM6 movement upon activation to the MII state. The model was prepared using the coordinates from the rhodopsin crystal structure (PDB F188,³⁸). Portions of the extracellular domain and intracellular loops have been removed for clarity. B) Spectra showing the spectral overlap of bimane emission with rhodopsin and MII absorption. The fluorescence emission spectrum of bimane shows much more overlap with dark state (DS) rhodopsin (shaded gray and black) compared to MII rhodopsin (black only). This results in more energy transfer from bimane to retinal in dark state rhodopsin. Because the amount of overlap is greatly reduced upon photoconversion to the MII state, a large increase in bimane fluorescence emission is observed due to relief of energy transfer. This increase must be taken into account when analyzing TrIQ data in rhodopsin studies. For more details, see^{54, 67}. C) The fluorescence from the bimane label on TM6 at site 250 increases in intensity upon MII formation, because there is less spectral overlap with retinal and thus less energy transfer (discussed above). D) In contrast, for the 139W/250B sample, MII formation is not accompanied by an increase in bimane emission intensity. This quenching is interpreted to be caused by the bimane at site 250 moving closer to the Trp at site 139, and thus undergoing TrIQ. For both the V250B and V139W/V250B samples, the same absorbance

spectra for the dark state and MII species were observed (not shown), ruling out optical artifacts due to other rhodopsin spectral species such as MIII. The steady-state fluorescence emission spectra were measured for rhodopsin mutants in 0.05% DM, 5 mM MES, pH 6.0 at 10 °C. MII conversion was achieved by photobleaching with > 490 nm light for 30 s (red), followed by the subsequent fluorescence scans. Figure adapted from ⁶⁷.