Rhodopsin Controls a Conformational Switch on the Transducin γ Subunit

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Summary
Rhodopsin, a prototypical G protein-coupled receptor, catalyzes the activation of a heterotrimeric G protein, transducin, to initiate a visual signaling cascade in photoreceptor cells. The βγ subunit complex, especially the C-terminal domain of the transducin γ subunit, Gtγ(60–71)farnesyl, plays a pivotal role in allosteric regulation of nucleotide exchange on the transducin α subunit by light-activated rhodopsin. We report that this domain is unstructured in the presence of an inactive receptor but forms an amphipathic helix upon rhodopsin activation. A K65E/E66K charge reversal mutant of the γ subunit has diminished interactions with the receptor and fails to adopt the helical conformation. The identification of this conformational switch provides a mechanism for active GPCR utilization of the βγ complex in signal transfer to G proteins.

Introduction
Interactions between the light receptor rhodopsin (R) and the G protein transducin (Gt) in rod photoreceptor cells represent one of the first major molecular events that lead to the generation of visual signals [1]. The βγ subunit complex of Gt is required for effective interactions between photoactivated rhodopsin (R*) and Gt and for the subsequent nucleotide exchange on the Gt α subunit [2]. The exact mechanism of this requirement is unclear. Traditionally, Gtβγ is assigned a membrane-targeting role, supported by the presence of hydrophobic posttranslational modifications of the Gt C terminus by isoprenoid farnesyl and carboxymethylation. This model views Gtβγ as a rigid and passive structure because of the lack of conformational changes between X-ray structures of Gtβγ and Gtβγ [3]. Recent expansion of this model gives Gtβγ a more prominent, possibly direct, role in nucleotide exchange on Gtα. Several lines of evidence support this new concept. (1) Gtα requires both R* and Gtβγ for effective nucleotide exchange, even in the absence of lipid membranes in vitro [4–6]. (2) Mutations, peptide competition, and biophysical studies have identified domains on both β and γ subunits of Gt that interact with R* [6–11]. (3) Mutations in the region of the Gtβγ subunit that are in contact with the switch regions of Gtα have been shown to affect Gtα activation by R* without interfering in αβγ subunit interactions [12]. These mutations and the well-known inhibitory effect of αβγ complex formation on spontaneous GDP release link Gtβγ to the regulation of a nucleotide exchange on Gtα. Finally, (4) analysis of the X-ray structures of small monomeric G proteins complexed with corresponding nucleotide exchange factors revealed that, in the Gt heterotrimer, Gtβγ occupies the position of a nucleotide exchange factor [13]. Together these pieces of evidence suggest active participation of Gtβγ in Gtα activation. We and others have identified a C-terminal region of Gtγ as a major domain for interactions with R* and as a possible conduit for R* to regulate Gtα [8, 9, 11, 14]. We have also presented biochemical evidence of a possible regulatory switch in this region induced by R* [14]. Despite these predictions, direct structural evidence of a conformational switch in the Gtβγ complex induced by R* is lacking. The Gtγ(60–71)farnesyl region is mostly disordered in X-ray structures of Gtβγ [2]. Here, we report the R*-bound structure of the Gt(60–71)farnesyl domain, DKNPFKELKGCC-farnesyl, and conformational changes in this region identified in the presence of different activation states of rhodopsin. We show that a specific charge reversal in the C-terminal domain of Gtγ and in mimetic peptides representing this region abolishes R*-Gt interactions and disables the conformational switch in Gtγ.

Results and Discussion
Mutations in the C-Terminal Domain of the Transducin γ Subunit Prevent Effective Rhodopsin–Transducin Interactions
In order to define the role of Gtγ in R* interactions and Gt activation, we targeted the Gtγ(60–71) domain by reversing the positions of neighboring amino acids. Such mutations tend to disrupt the secondary structure of local domains, while preserving the amino acid composition, total charge, and hydrophobicity. We found that a mutant with reversed amino acid positions 65 and 66, the K65E/E66K charge-reversal mutant Gtβγ-KEr (Figure 1), is defective in supporting interactions with R*.

Gt interacts with rhodopsin in a light- and nucleotide-dependent fashion [4, 15]. In the dark, these interactions are weak. Photoactivation leads to the binding of Gt-GDP to R*, dissociation of GDP, and formation of a high-affinity R*-Gt-empty complex. Addition of GTP results in dissociation of active Gt-GTP from R* and lipid membranes and of Gtα-GTP from Gtβγ. Both Gtα and Gtβγ are absolutely dependent on each other for these high-affinity interactions with R*. At a fixed concentration of Gtα, binding to R* occurs in a Gtβγ concentration-dependent manner (Figure 1). With Gtβγ-KEr, the interactions between R* and Gt are significantly weakened and observed only at saturating concentrations. This effect is consistent with studies demonstrating that mutations in the C terminus of Gtγ do not affect G protein subunit interactions [14, 16] and that charge reversal

Key words: GTP binding protein; γ subunit; G protein-coupled receptor; rhodopsin; transducin
The amplitude of this additional, or extra, Meta II signal can be measured as the A380/A417 absorbance difference by UV/visible spectroscopy [6]. A model peptide that represents the surface domain of Gt, Gtγ(60–71)farnesyl, interacts with R* directly to stabilize Meta II (Figure 2A). When the KEr mutation is introduced into Gtγ(60–71)farnesyl, the peptide fails to stabilize Meta II (Figure 2A). Considering the expected differences in the microenvironment around the model peptide and the Gtγ(60–71) domain in the heterotrimer, this result is in remarkable agreement with the reduced ability of Gtγ/H9252/H9253-KEr to interact with R*. If signal transfer from R* to Gt does indeed proceed via a conformational switch in the C terminus of Gtγ, the charge reversal may disable the switch and prevent Gtγ activation.

In order to test this hypothesis, we have determined the three-dimensional structures of Gtγ(60–71)farnesyl and Gtγ(60–71)farnesyl-KEr in the R*–bound state by high-resolution proton TrNOESY and have studied the conformational dynamics of this region in the presence of different activation states of rhodopsin.

Figure 1. Binding of Gt Subunits to R*
Circles, wild-type Gtβγ; diamonds, Gtβγ complex with charge reversal at positions K65 and E66 in Gtγ. Error bars show the results from two independent experiments.

TrNOE Structures of the R*–Bound C-Terminal Domain Gtγ(60–71)farnesyl
TrNOE proton spectra of Gtγ(60–71)farnesyl and Gtγ(60–71)farnesyl-KEr were recorded in the presence of dark-adapted inactive rhodopsin, photoactivated rhodopsin (R*), and inactivated rhodopsin (opsin plus all-trans-retinal) after complete Meta II decay (Figure 2B). The same sample was used to collect all data; thus, photoactivation of rhodopsin was the only variable in these experiments.

The TrNOE spectra of the Gtγ(60–71)farnesyl domain in the presence of inactive R showed good chemical shift dispersion in the NH–NH, NH–αH, and the majority of NH–aliphatic regions. Total correlation spectroscopy (TOCSY) [20] and NOESY [21] were used to generate sequence-specific and stereo-specific assignments. All sequential connectivities in the signature region (NH–αH) are easily observed, except for the P63–N62 pair because of the lack of NH in proline (Figure 3A). All sequential NOE peaks in the NH–NH region were observed but were very weak. Sequential proton cross-relaxations were evident in the NH–aliphatic and aliphatic–aliphatic regions. Protons of the farnesyl group were assigned, and a short-range connectivity between protons of the terminal C1 of farnesyl and CH3 of the side chain of C71 were observed. Analysis of the dark spectra revealed that, except for the weak F64–L67 side chain NOEs, no NOE crosspeaks that would represent medium- or long-range interactions within the Gtγ(60–71)farnesyl domain were present, suggesting a high degree of disorder in this domain in the presence of inactive R. These data are also consistent with the disordered conformation of the C-terminal domain of Gtγ in all X-ray structures of Gt.

Photoactivation resulted in dramatic changes in the TrNOE spectra. The overall intensity diminished slightly, and the NOE peaks broadened marginally, reflecting the interactions of Gtγ(60–71)farnesyl with R*. Proton chemical shifts did not change significantly, allowing direct comparison of the NOE spectra before and after
activation. The full set of more-defined sequential NH–NH crosspeaks was observed. Interresidue and short-range NH–aliphatic interactions for K61, K65, E66, and K68 became more prominent, indicating a certain ordering of Gt\textsubscript{$\gamma$}(60–71)farnesyl upon binding to R*. Thirty-two additional medium- to long-range NOE peaks were identified in the R*-bound state. Representative changes in the NH–H region are shown in Figure 3A, and a full NOE summary is shown in Figure 3B. Several interactions important for structure calculations were identified in the NH–H region, including N62-F64(NH,NH i,i+2), P63-K65(NH,NH i,i+2), F64-K68(NH,NH i,i+4), E66-K68(NH,NH i,i+2), and L67-G70(NH,NH i,i+3). Analysis of the NH–NH region revealed additional N62-F64(NH,NH i,i+2), L67-G69(NH,NH i,i+2), and K68-G70(NH,NH i,i+2) NOE peaks. Importantly, all photoactivation-specific NOEs disappeared after 24 hr, as Meta II decayed to opsin and all-trans-retinal. These data strongly argue for a defined R*-bound conformation of the Gt\textsubscript{$\gamma$}(60–71)farnesyl domain, while it appears disordered before activation and after Meta II inactivation.

TrNOE spectra of the Gt\textsubscript{$\gamma$}(60–71)farnesyl-KEr recorded under the same experimental conditions did not reveal any activation-dependent differences (data not shown). The lack of meaningful medium- and long-range NOEs signifies a disordered state before and during rhodopsin activation, in striking contrast to the conformational changes in the native domain.

Three-dimensional structures of R*-bound Gt\textsubscript{$\gamma$}(60–71)farnesyl based on NMR-derived constraints were calculated by distance geometry, constrained high-temperature molecular dynamics, and simulated annealing.

Figure 3. NMR Spectra of Gt\textsubscript{$\gamma$}(60–71)farnesyl in R*-Bound State
(A) Representative NMR spectra of Gt\textsubscript{$\gamma$}(60–71)farnesyl after photoactivation of R. The solid red line follows sequential NH–H interactions. Activation-specific NOE peaks, green.
(B) A summary of observed NOEs for R*-bound Gt\textsubscript{$\gamma$}(60–71)farnesyl. The H bonds were identified during structure calculations.
Fifteen NMR structures were computed and superimposed with an rmsd of 0.59 Å for main chain atoms and 1.75 Å for side chain atoms. Ramachandran plot statistics and residue properties confirm that the models are geometrically valid (Figure 4B). A ribbon diagram of a representative structure is shown in Figure 4A. Binding of the Gt\(\text{y}(60–71)\)farnesyl domain to R* leads to the formation of an amphipathic helix, with F64 and L67 representing a hydrophobic surface, while K61, K65, and K68 form a strong hydrophilic surface (Figure 4). The helical model of Gt\(\text{y}(60–71)\)farnesyl in the R*-bound state is based on a complete set of sequential NH-NH connectivities, observed medium- to long-range interactions, including unique NH-NH \(i,i+2\), \(\alpha\text{H,NH }i,i+2\), \(\alpha\text{H,NH }i,i+3\), and \(\alpha\text{H,NH }i,i+4\) peaks, and strong side chain interactions of an \(i,i+3\) type (Figure 3B). In addition, a full set of \(i,i+4\) main chain H bonds have been identified between K65 and C71 during structure calculations, which provide additional stabilization of the helix. In order to confirm that the NOE data set of the Gt\(\text{y}(60–71)\)farnesyl peptide in the R*-bound form defines a helical conformation, we have repeated high-temperature molecular dynamics and simulated-annealing calculations with NOE constraints removed. As expected, under these control conditions, heating the structures to 1000 K results in quick deterioration of the helical elements and complete disorganization of this small peptide. Overall, both experimentally determined restraints and calculated structures point to a well-defined helical conformation of the Gt\(\text{y}(60–71)\)farnesyl stretch in the R*-bound state. KE charge reversal appears to disrupt the amphipathic nature of the helix, making the structure unstable, even in the presence of R*. The transition of the Gt\(\text{y}(60–71)\)farnesyl conformation from disordered to helical constitutes a molecular switch controlled by rhodopsin.

Analysis of the NOE pattern reveals some heterogeneity with regard to the NOEs typically observed in \(\alpha\) helices (\(\alpha\text{H,NH }i,i+4\) and \(\alpha\text{H,NH }i,i+3\)), versus NOEs characteristic of a 3(10) helix (\(\alpha\text{H,NH }i,i+2\) and \(\alpha\text{H,NH }i,i+3\)). Several explanations can account for the presence of the \(\alpha\text{H,NH }i,i+2\) interactions in what could otherwise be considered a set consistent with an ideal \(\alpha\) helix. Interpretation of the NOE signals can be complicated by competing experimental requirements (see Experimental Procedures). Without knowledge of the precise geometry of a complementary binding site on R*, cross-relaxation between R* and Gt\(\text{y}(60–71)\)farnesyl or indirect magnetization transfer effects cannot be completely excluded as the source of observed \(\alpha\text{H,NH }i,i+2\) interactions and may indicate slow exchange between bound and free peptide. If \(\alpha\text{H,NH }i,i+2\) NOEs are artifactual, the R*-bound conformation is \(\alpha\) helical, with slight distortion of ideal geometry around P63. An alternative explanation of NOE heterogeneity may come from the dynamic nature of TrNOESY experiments, when the ligand [Gt\(\text{y}(60–71)\)farnesyl] is in exchange between the R*-bound and unbound state. Continuous transitions from a disordered to an \(\alpha\)-helical conformation via 3(10) helical turns would be typical and expected [22–24], resulting in certain conformational averaging. Finally, it is possible that the R*-bound state may be represented by a number of conformations, ranging from an ideal \(\alpha\) helix to an ideal 3(10) helix. Elements of both substructures would be expected to manifest themselves in the NMR experiments. Computer simulations show that the interconversion between \(\alpha\) and 3(10) helix in a hydrophobic environment requires less than 6 kcal/mol in free energy for a model decapetide [22]. Therefore, the bound conformation of the \(\gamma\) peptide in a hydrophobic microenvironment of the R* binding site may be populated by both \(\alpha\) and 3(10) conformations of what has been termed a molten helix [22].

Mechanism of a Conformational Switch in Gt\(\text{y}^-\)
To visualize the conformational changes of the Gt\(\text{y}(60–71)\)farnesyl region in the context of the heterotrimeric
Helix-destabilizing KE charge reversal disables this switch in the corresponding amino acid sequences of different subtypes of Gtγ subunits (m, mouse; h, human; b, bovine; ce, C. elegans; d, D. melanogaster). Conserved amino acids are highlighted in yellow.

C-terminal of Gtγ in the R*-bound state effectively shortens the distance between the two C-terminal domains, making the receptor binding site on Gt more compact.
More importantly, the G\textsubscript{T}\gamma switch identified in this study provides a mechanism for active G\textsubscript{PCR} utilization of the G\textsubscript{T}\gamma complex in signal transfer to G proteins.

**Experimental Procedures**

**Purification of Rhodopsin and Gt Subunits**
Rod outer segments were prepared by the method of Papamaster and Dreyer [28], and urea-washed membrane procedures were adapted from Yamazaki et al. [29] and Willardson et al. [30], as we described [31]. Gt was purified by GTP elution from isotonically washed ROS disks, and Gt\textsubscript{\gamma} and Gt\textsubscript{T}\gamma were separated by AKTA FPLC on Blue Sepharose CL-6B.

**Expression and Purification of Transducin Mutants**
Gt\textsubscript{\gamma} mutants were constructed by PCR. Baculoviruses were produced with the Bac-to-Bac Baculovirus Expression System (Gibco BRL). Sf9 cells were coinfected with Gt\textsubscript{\gamma} and Gt\textsubscript{T}\gamma-His\textsubscript{6}, viruses at predetermined ratios. Purification was done on an N-NTA Superflow (QIAGEN). Protein purity was confirmed by SDS-PAGE.

**Synthesis and Purification of Gt\textsubscript{\gamma}(60–71)farnesyl**
Peptide synthesis, prenylation, purification, and mass spectrometry were described [8]. The peptides were synthesized on an Applied Biosystems solid-phase peptide synthesizer by Fmoc chemistry and reacted with farnesyl bromide (Aldrich) and then purified on a reverse-phase AKTA FPLC. The carboxy-terminal cysteine is not carboxymethylated to improve solubility in aqueous solutions.

**Rhodopsin Binding**
Various amounts of the Gt\textsubscript{T}\gamma were reconstituted with 0.5 \mu M of Gt\textsubscript{\gamma} and 5 \mu M of urea-washed ROS membranes, UMs, in buffer R, 10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 3 mM MgCl\textsubscript{2}, 1 mM EDTA, 1 mM DTT, 10 \mu M PMSF, and 2 \mu g/ml leupeptin. The reaction was initiated by exposure to light. UMs were centrifuged at 150,000 \times g and 4°C for 15 min in a TLA-100.3 rotor on a Beckman TL-100 Ultracentrifuge. The pellet was washed with buffer R. A UM with Gt bound was resuspended in 10 mM Tris-HCl (pH 8.0), 3 mM MgCl\textsubscript{2}, 1 mM EDTA, 1 mM DTT, and 100 \mu M GTP-S, incubated at 4°C for 30 min, and centrifuged. The supernatant was analyzed for the presence of G protein subunits by immunoblotting. Concentrations of Gt\textsubscript{T}\gamma and Gt\textsubscript{\gamma}-K\textsubscript{\epsilon} were standardized prior to the assay.

**Meta II Stabilization**
Extra Meta II was modified by difference UV/visible spectroscopy on a Varian Cary 50, essentially as described previously [6, 9]. Measurements were taken in 100 mM Na-HEPES (pH 7.9), 50 mM NaCl, 1 mM DTT, 1 mM MgCl\textsubscript{2}, 1 mM EDTA at 4°C, 10 \mu M rhodopsin, and various amounts of peptides. Meta II was calculated as the A380/A417 difference.

**TrNOE Spectroscopy and Structure Calculations**
One-dimensional and high-resolution 2D proton spectra were acquired on a Varian Unity 600 with VNMRF 5.2 at 4°C as described [18]. Samples contained 0.1 mM rhodopsin as a fine suspension of native urea-washed rod outersegment membranes with a purity of 99.5%, 2 mM of Gt\textsubscript{T}\gamma(60–71)farnesyl or Gt\textsubscript{\gamma}(60–71)farnesyl-K\textsubscript{\epsilon} in 10 mM Na-phosphate (pH 7.0), and 10% D\textsubscript{2}O. The estimated T\textsubscript{1}\textsubscript{\epsilon} of R* under the NMR conditions is 110 min. TOCSY utilized the MLEV-17 mixing sequence of 120 ms, flanked by two 2 ms trim pulses, a 0.5 s preacquisition delay, and a 1.0 s presaturation. To obtain an optimal signal to noise ratio for NOEs while minimizing possible indirect magnetization transfer in the system, we considered a range of concentrations of rhodopsin membranes and various NOE mixing times. The working concentration of the peptide at 2 mM is necessary for complete stabilization of the active Meta II and is below the concentration range at which any aggregation of the peptide can be seen. The concentration of rhodopsin membranes was determined by titration and monitoring the 1D spectra of the peptide for line width broadening. Only slight broadening was detected for rhodopsin concentrations up to 0.1 mM. In a set of preliminary NOESY experiments, a range of T\textsubscript{1}\textsubscript{\epsilon} from 150 ms to 400 ms was explored. The NOE buildup was linear for a T\textsubscript{1}\textsubscript{\epsilon} of up to 300 ms. The final T\textsubscript{1}\textsubscript{\epsilon} was 250 ms. A 2 \times 280 \times 2049 data matrix with 16 scans per t\textsubscript{1} was used for NOESY. The water suppression protocol was WATERGATE. NOEs were classified into weak, medium, and strong, with interproton distances of 1.9–5.0 Å, 1.9–3.5 Å, and 1.9–2.7 Å. Under experimental conditions of excess ligand, typical for a TrNOESY setup, sequential NOEs from the unbound ligand create background signals that obscure the true intensities of the sequential NOEs in a bound state. In order to minimize potential errors caused by overestimating NOE intensities, we tabulated most of the additional NOE peaks as weak, with a corresponding distance range of 1.9–5.0 Å, and the sequential NOEs as medium, with a corresponding distance range of 1.9–3.5 Å. An initial set of structures was generated by distance geometry with DISTGEOM of TINKER 3.9 and CHARM22 forcefield [18, 32]. Farnesyl was omitted from calculations. Structures with the least violations of distance restraints were subjected to restrained molecular dynamics at 1000 K and simulated annealing (0.5 ps time steps, 10 ps total time). Fifteen structures were computed independently and superimposed in MOLEMOL [33]. In Gt\textsubscript{T}\gamma (Protein Data Bank accession number 1AOR), missing C-terminal residues were rebuilt in INSIGHT II, connected to farnesyl, and energy minimized to produce ground conformation. For the R* bound state, a representative TrNOESY structure was fused to Gt\textsubscript{T}\gamma via overlap in 60–65 and connected to the farnesyl at the C terminus, and then local energy minimization was performed.

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**References**


Accession Numbers

The atomic coordinates of an ensemble of fifteen structures have been deposited in the Protein Data Bank under ID code 1MF6.