Conformational Changes Associated with Receptor-stimulated Guanine Nucleotide Exchange in a Heterotrimeric G-protein α-Subunit

NMR ANALYSIS OF GTPγS-BOUND STATES

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Solution NMR studies of a 15N-labeled G-protein α-subunit (Gα) chimera (15N-ChiT)-reconstituted heterotrimer have shown previously that G-protein βγ-subunit (Gβγ) association induces a “pre-activated” conformation that likely facilitates interaction with the agonist-activated form of a G-protein-coupled receptor (R*) and guanine nucleotide exchange (Abdulaev, N. G., Ngo, T., Zhang, C., Dinh, A., Brabazon, D. M., Ridge, K. D., and Marino, J. P. (2005) J. Biol. Chem. 280, 38071–38080). Here we demonstrated that the 15N-ChiT-reconstituted heterotrimer can form functional complexes under NMR experimental conditions with light-activated, detergent-solubilized rhodopsin (R*), as well as a soluble mimic of R*. NMR methods were used to track R*-triggered guanine nucleotide exchange and release of guanosine 5′-O-3-thiotriphosphate (GTPγS)/Mg2+-bound ChiT. A heteronuclear single quantum correlation (HSQC) spectrum of R*-generated GTPγS/Mg2+-bound ChiT revealed 1HN, 15N chemical shift changes relative to GDP/Mg2+-bound ChiT that were similar, but not identical, to those observed for the GDP-AIF7/Mg2+-bound state. Line widths observed for R*-generated GTPγS/Mg2+-bound 15N-ChiT, however, indicated that it is more conformationally dynamic relative to the GDP/Mg2+- and GDP-AIF7/Mg2+-bound states. The increased dynamics appeared to be correlated with Gβγ and R* interactions because they are not observed for GTPγS/Mg2+-bound ChiT generated independently of R*. In contrast to R*, a soluble mimic that does not catalytically interact with G-protein (Abdulaev, N. G., Ngo, T., Chen, R., Lu, Z., and Ridge, K. D. (2000) J. Biol. Chem. 275, 39354–39363) is found to form a stable complex with the GTPγS/Mg2+-exchanged heterotrimer. The HSQC spectrum of 15N-ChiT in this complex displays a unique chemical shift pattern that nonetheless shares similarities with the heterotrimer and GTPγS/Mg2+-bound ChiT. Overall, these results demonstrated that R*-induced changes in Gα can be followed by NMR and that guanine nucleotide exchange can be uncoupled from heterotrimer dissociation.

G-protein coupled receptors (GPCRs) represent a diverse group of seven transmembrane (TM) helix receptors that require agonist-dependent activation to initiate heterotrimeric (αβγ) G-protein-mediated intracellular signaling cascades. GPCR activation of cognate G-proteins are the first steps in cellular communication pathways responsible for signaling cascades that mediate vision, olfaction, taste, and the action of numerous hormones and neurotransmitters (1). Activation of a G-protein by its agonist-stimulated GPCR (R*) requires the propagation of structural signals from the receptor-binding interface to the guanine nucleotide-binding pocket. The structural basis for the interaction of a GPCR with its cognate G-protein and the subsequent activation of the G-protein by R* is not fully understood.

Using signaling of the retinal G-protein transducin (Gt) by rhodopsin as a model system, we are applying solution NMR methods to track changes in the G-protein α-subunit (Gα) associated with activated R* interactions. Rhodopsin, the rod cell photoreceptor involved in dim-light vision, represents one of the best studied GPCRs in terms of structure and function (2, 3). Photon capture triggers cis → trans isomerization of the retinal chromophore, which initiates structural changes in the TM helices resulting in the formation of the light-activated signaling state metarhodopsin II, R*. This is accompanied by functionally significant changes at the cytoplasmic surface that leads to the formation of binding and activation sites for several signaling proteins, including Gα (4–10). Crystal structures for the inactive (dark) state of rhodopsin have provided a detailed view of the retinal binding site and the cytoplasmic region (11–15). Although remarkably informative, the crystal structures provide few solid insights into the mechanism of signal transfer from R* to Gα.

Binding of heterotrimeric G-proteins to activated GPCRs requires the presence of both Gα and G-protein βγ-subunits (Gβγ). The following three regions on the α-subunit of Gα (Gαα) are known to be important for receptor interactions; the amino-terminal 23 residues, an internal sequence from amino acids 305–315, and the carboxyl-terminal 11 amino acids (16–18). Upon binding to R*, Gαα is thought to undergo structural changes in both the amino- and carboxyl-terminal regions. High resolution crystal structures of Gαα subunits, including Gαα (19–24), Gβγ (25), and Gαα heterotrimeric complexes (Refs. 26 and 27, Fig. 1A), have provided important insights into the structural rearrange-
ments accompanying guanine nucleotide exchange and the GTPase cycle, particularly in the conformationally flexible switch regions. In most of the crystal structures, however, the residues at the extreme carboxyl terminus of Gα are disordered and/or not visible. This is consistent with findings from transferred nuclear Overhauser enhanced spectroscopy NMR studies (28–30) on 11 amino acid carboxyl-terminal peptides derived from Gia (Gia (340–350) peptides), which show that these peptides are largely unstructured in solution and in the presence of dark state rod outer segment (ROS) rhodopsin, but undergo significant structural changes upon binding to light-activated rhodopsin. Similarly, the helical structure of the amino terminus of Gα appears transient and is only ordered in crystal structures of the heterotrimer (26, 27). Results from a study in which fluorescent and spin-label probes were introduced at specific positions in the amino-terminal region of Gα are consistent with the amino terminus by assuming an ordered helical conformation only in Gia (31).

High resolution structural analysis of R*G-protein interactions poses many significant challenges given the inherently dynamic nature of this process. The R*G interaction can be viewed as taking place in at least five discrete biochemical reaction steps (Fig. 1B). These include R* binding to Gia,GDP to form the R*Gia,GDP complex (steps 1 and 2), GDP dissociation from the R*Gia,GDP complex to form an R*Gia,empty complex (step 3), GTP uptake by the R*Gia,empty complex to form R*Gia,GTP (step 4), and dissociation of Gia,GTP from R* followed by Gia,GTP from Gia (step 5), with R* now free for interaction with another Gia,GTP (32). Although the above mentioned crystallographic studies have been instrumental for obtaining static three-dimensional structures of dark state rhodopsin and various guanine nucleotide-bound states of Gα, and biochemical and mutational approaches have provided a wealth of information about the nature of R*G interaction, a structural representation of the R*-Gα complex(es) remains poorly defined. Clearly, a comprehensive description of the structures involved in these reaction steps would provide important insights into the mechanisms governing activated GPCR/G-protein interactions.

We have shown previously that a Gα chimera consisting of sequences from Gia and Gia (Chie; see Ref. 33) can be expressed to high levels in a soluble form by using a subtilisin promdomain (proR8FKAM) fusion construct and milligram quantities of promdomain-released, full-length, isotope-labeled Gα (Chie) purified in a single step by using an immobilized "slow cleaving" mutant form of subtilisin (34). This has allowed us to pursue functional studies under NMR experimental conditions that provide insights into the solution structures of Gα in various states. We have also shown that isotope-labeled Chie can be reconstituted with Gia subunits to form a functional heterotrimer that is amenable to structural analysis by high resolution NMR (35). This latter work revealed that Gβγ binding to Chie induces structural changes in the guanine nucleotide binding and carboxyl-terminal regions of Chie, leading to a "preactivated" state that may facilitate interaction with R* and subsequent GDP/GTP exchange. Here we have now applied high resolution NMR to begin to probe the structural basis for the propagation of signals from R* to the G-protein, with the specific goal of developing more robust models for the structural changes in Gα that accompany the signal transfer process. Specifically, NMR methods have been used to track the complete cycle of guanine nucleotide exchange in 15N-ChiT-reconstituted heterotrimer that is triggered by light-activated rhodopsin (R*), thereby providing new insights into Gα conformational changes-associated signal propagation from an activated GPCR. Using similar NMR approaches, guanine nucleotide exchange in a 15N-ChiT-reconstituted heterotrimer stimulated by a soluble mimic of R* has been monitored. In contrast to R*, the soluble mimic remains bound to the nucleotide-exchanged heterotrimer forming a trapped, stable complex akin to the R*Giaβγ,GTP intermediate in the reaction pathway.

**EXPERIMENTAL PROCEDURES**

*Materials—Cyclohexylpentyl-β-D-maltoside (Cymal-5) and n-dodecyl β-D-maltopyranoside (DM) were from Anatrace. GTPγS was from Roche Applied Science, and Ni2+-nitrilotriacetic acid-agarose resin was from Qiagen. [35S]GTPγS was from PerkinElmer Life Sciences. Anti-Gia and anti-Gia antibodies were from Affinity BioReagents, and the anti-rhodopsin antibody K42–41L (37) was a gift from Prof. Paul Hargrave (University of Florida). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies were from Santa Cruz Biotechnology, and protein G-Sepharose was a gift from Prof. Philip Bryan (University of Maryland Biotechnology Institute). The pG58 expression vector, a fusion vector encoding a modified 77-aminocaproic acid promdomain region of subtilisin BPN' (proR8FKAM), and the pG58-derived expression vector encoding a Gα chimera (Chie) as a proR8FKAM fusion have been described (34, 36). The sources of other materials used in this investigation have been reported (34, 35, 38).*

**Expression and Purification of Subtilisin Promdomain/Chie Fusions—** Methods for the inducible bacterial expression and purification of isotope-labeled wild-type and mutant Gie using the proR8FKAM/Chie fusion and immobilized S189 subtilisin BPN' have been described (34). To generate the GTPγS/Mg2+-bound form of Chie independently of R*, GDP was omitted from the cell lysis and column purification buffers in order to obtain an ‘empty pocket’ state of Gie that could subsequently be reconstituted with GTPγS. Prior to NMR analysis, the purified and isotope-labeled proteins were concentrated and dialyzed against 25 mM d1,Tris-HCl, pH 7.5, containing 100 mM NaCl, 5 mM magnesium acetate, 2.5 mM dithiothreitol, and 5% glycerol (Buffer A).

**Expression and Purification of HPTRX/CDEF—** Detailed protocols for the inducible expression and purification of HPTRX/CDEF, a soluble mimic of R*, have been described (38). Prior to NMR experiments, purified HPTRX/CDEF was concentrated and dialyzed against Buffer A.

**Detergent Solubilization and Purification of Rhodopsin—** ROS rhodopsin from bovine retina was solubilized and purified in Cymal-5 or DM detergent on rho-1DA-Sepharose essentially as described (39, 40). Rhodopsin concentrations were determined by UV-visible spectrophotometry at 20 °C using a λ25 spectrophotometer (PerkinElmer Life Sciences). Prior to NMR experiments, rhodopsin preparations were concentrated and dialyzed against Buffer A containing 0.08% (w/v) Cymal-5.

**Filter Binding Assay for Measuring G-protein-mediated Guanine Nucleotide Exchange—** The ability of detergent-solubilized rhodopsin preparations to catalyze the uptake of [35S]GTPγS by Gie was determined by initial rate analysis, and at equilibrium, using a nitrocellulose filter binding assay essentially as described (41). For initial rate analyses, the reaction mixtures contained 6.7 nM Cymal-5 or DM-solubilized and -purified ROS rhodopsin and 5 μM [35S]GTPγS in 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 5 mM MgCl2, and 2.5 mM dithiothreitol (Buffer B). After illumination (>495 nm) for 1 min at 20 °C, the reactions were initiated by the addition of 4 μM Gie. The total reaction volume was 250 μL, and the final concentrations of Cymal-5, DM, and glycerol in the assay mixtures were 0.08, 0.015, and 5% (w/v), respectively. At various time intervals (5–20 s), a 50-μL aliquot was removed, rapidly filtered through nitrocellulose with the aid of a vacuum manifold, and the filters immediately washed three to four times with 5 ml of Buffer B to remove free, unbound [35S]GTPγS. The filters were dried,
and the G-protein-bound [35S]GTPγS was determined by scintillation counting. For the equilibrium assays, the same reaction mixture containing 4 μM Gt was illuminated for 1 min, the reaction allowed to proceed for 2 h at 20 °C, and the extent of GDP/GTPγS exchange determined as described above. Identical reactions were performed in the dark for both the initial rate and equilibrium assays. The activity in the dark was subtracted from that in light for determination of the kinetic and equilibrium values, which are reported as averages ± S.E.

Rate of Metarhodopsin II (R*) Decay in the Absence and Presence of Gt—
The rate of retinal release upon R* decay in 0.08% Cymal-5 or 0.015% DM, and in the presence Gt was determined by following the decrease in protonated retinyl-Schiff base as measured at 440 nm after acidification essentially as described (42–44). Briefly, purified rhodopsin (1.17 μM) in 25 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 5 mM magnesium acetate, 2.5 mM dithiothreitol, 5% glycerol (Buffer C), and 0.08% Cymal-5 or 0.015% DM was illuminated (>495 nm for 1 min) at 20 °C in the absence or presence of Gt (1.4 μM, 1.2-fold excess over rhodopsin). Aliquots (400 μl) were removed at specific time points (typically at 1, 15, 30, 60, 120, 240, and 480 min) and acidified to pH 3 by addition of 2 N H3SO4. After mixing, a UV-visible spectrum (650 nm-250 nm) was recorded, and the amount of the protonated retinyl-Schiff base (440 nm) remaining as a function of time was determined.

Reconstitution of ChiT with Gtβγ to Form the Gtβγ, Heterotrimer—
The G-protein heterotrimer was reconstituted from isotope-labeled ChiT and Gtβγ essentially as described (35). Prior to NMR experiments, heterotrimer preparations were concentrated and dialyzed against Buffer A or Buffer A containing 0.08% Cymal-5.

Immunoprecipitation and Analysis of the Gtβγ,GTPγSHPRTX/CDEF Complex—Equinorm concentrations of HPRTX/CDEF and ChiT-reconstituted heterotrimer (500 nM) in Buffer C were mixed with GTPγS (600 nM) and incubated for 30 min at 20 °C. An aliquot of anti-Gtβγ antibody (20 μl of a 1 mg/ml solution) was added to the mixture, followed by 200 μl of pre-cleaned protein G-Sepharose. After gentle mixing for 30 min at 20 °C, the beads were allowed to settle, the supernatant removed, and the beads washed five times with 1 ml of Buffer C. The washed beads were resuspended in reducing SDS-PAGE sample buffer (250 μl), and aliquots (50 μl) were examined by reducing SDS-PAGE (45) using a 5% stacking and a 16% resolving gel. The immunoprecipitated proteins were electroblotted onto poly(vinyl difluoride) membranes (46), detected with the K42–41L (HPRTX/CDEF), anti-Gtα (ChiT), and anti-GtβGtδ (Gtδ) antibodies, or a mixture of these primary antibodies, and horseradish peroxidase-conjugated goat anti-mouse and/or anti-rabbit antibodies. The proteins were visualized by chemiluminescence.

NMR Spectroscopy—One-dimensional 1H- and 15N-filtered 1H water flip-back, water gate, 15N-decoupled spectra and two-dimensional 15N-HSQC water flip-back, water gate spectra (47) were acquired at 30 °C using a Bruker AVANCE 600-MHz spectrometer (Bruker Instrum., Billerica, MA) equipped with a triple-resonance 1H, 13C, 15N axis gradient cryoprobe and linear amplifiers on all three channels. Spectra were collected on uniformly 15N-labeled samples (15N-ChiT) dissolved in Buffer A at concentrations of 150–300 μM. The nitrogen frequency was centered at 118 ppm and the proton frequency on H2O (~7.5 ppm). One-dimensional spectra were collected using a sweep width of 7,200 Hz and 2,048 complex points, and two-dimensional data were acquired using sweep widths of 7,200 Hz in 2Dω and 2,048 Hz by 64 complex data points in t2 and t1, respectively, (t1(max) = 293 ms and t2(max) = 64 ms) and 128 scans per increment. NMR samples containing rhodopsin were placed in the spectrometer under dim-red light conditions. To initiate the guanine nucleotide exchange reaction, rhodopsin was illuminated with >495 nm light for 1 min prior to spectral acquisition. For NMR samples containing HPRTX/CDEF, the protein was added directly to the reaction mixture to stimulate guanine nucleotide exchange. All spectra were processed and analyzed on a SGI UNIX work station using NMRPipe (48). Trp indole and Phe-350 amide 1H and 15N resonances were assigned using ChiT mutants as described previously (34, 35). The aluminum fluoride (AlF4-) adduct of GDP/Mg2+bound ChiT was formed by addition of NaF (10 mM) and AlCl3 (300 μM) directly to the NMR tube.

Other Methods—A fluorescence assay for monitoring Gt activation by HPRTX/CDEF was performed essentially as described (38). Protein determinations were done as described previously (49).

RESULTS

Experimental Design and General Considerations—Having previously demonstrated that 15N-ChiT can be functionally expressed as a subtilisin BPN′ prodomain fusion and purified on immobilized S189 subtilisin BPN′, reconstituted with unlabeled Gtβγ subunits to form a heterotrimer (~85 kDa), and characterized by high resolution NMR (34, 35), it was of keen interest to now investigate whether NMR could
also be used to follow some of the reaction steps involved in R*-catalyzed guanine nucleotide exchange (Fig. 1B). Because these solution NMR studies would necessitate the use of rhodopsin, an ~40-kDa light-sensitive integral membrane protein, it was necessary to identify a detergent that could not only effectively solubilize rhodopsin but also support the formation of R* at concentrations below the critical micelle concentration (CMC). The rationale for choosing such a detergent for these NMR studies was as follows. At detergent concentrations below the critical micelle concentration (CMC), rhodopsin would be expected to be in micelles, which could contain several randomly oriented molecules and approach several hundred kDa. Such micelles would tumble extremely slowly and result in significant additional line broadening and relaxation effects. Interpretation of the NMR spectra for this case would represent an additional technical challenge. Therefore, we expended a considerable effort in identifying such a detergent, and we found that Cymal-5 (5-cyclohexyl-1-pentyl-β-D-maltoside), a relative of the commonly used rhodopsin-solubilizing detergent DM, appears to fulfill the necessary criteria (see below). The CMC of Cymal-5 has been determined to be 2.4 mM (~0.12%) in aqueous buffers (50), and its readily dialyzable nature enables facile manipulation of sample detergent concentration for NMR measurements.

**Properties of Rhodopsin and R* in Cymal-5 Detergent**—Representative UV-visible absorption spectra of immunopurified ROS rhodopsin in 0.08% Cymal-5 are shown in Fig. 2A. The Cymal-5-solubilized and -purified rhodopsin exhibits the characteristic 500 nm dark state chromophore and shows a shift in A$_{\text{max}}$ to ~380 nm upon illumination. Subsequent acidification of this photoproduction yields a 440-nm absorbing species, which is characteristic of a protonated retinyl-Schiff base, and suggests that the 380-nm absorbing species most likely represents the metarhodopsin II photointermediate R*. In general, these spectral transitions are qualitatively similar to those observed upon similar treatment of purified ROS rhodopsin in DM (51), suggesting that Cymal-5 does not adversely affect the structural integrity of the pigment.

Previous guanine nucleotide exchange assays have shown that purified rhodopsin in 0.08% Cymal-5 also catalyzes the light-dependent uptake of GTP$_\gamma$S by Gt, and the ChiT-reconstituted heterotrimer (35). A more detailed analysis of the kinetics of Gt activation by Cymal-5 purified light-activated rhodopsin showed that the rate of GDP/GTP$_\gamma$S exchange was nearly indistinguishable from that obtained for DM-purified rhodopsin (Fig. 2B). In both cases, ~3 mol of GTP$_\gamma$S were exchanged per second at 4 μM Gt (2.9 ± 0.23 (n = 3) and 3.1 ± 0.32 (n = 3) for Cymal-5 and DM-solubilized ROS rhodopsin, respectively). In contrast, equilibrium assays showed that the level of GTP$_\gamma$S exchanged after exhaustive binding was greater for R* in Cymal-5 than in DM, each catalyzing on average the exchange of ~324 ± 21.4 (n = 3) and ~262 ± 17.3 (n = 3) mol at 4 μM Gt, respectively.

The rate of R* decay in 0.08% Cymal-5 was also examined in the absence and presence of Gt, and with that for R* in 0.015% DM, a concentration just above the CMC of this detergent in aqueous buffer (50). As shown in Fig. 2C, purified rhodopsin in 0.08% Cymal-5 shows the characteristic loss of 440 nm absorbance as a function of post-illumination time. After 8 h at 20°C, only ~40% of the protonated retinyl-Schiff base remained, indicating that the majority of R$^*$ had decayed to opsin and free all-trans-retinal. The $t_{1/2}$ of this process was
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~28 min. For rhodopsin in 0.015% DM, ~70% of the R* decayed to opsin and free all-trans-retinal over the 8-h time course with a t₁/₂ of ~15 min, a half-life in agreement with the previous findings of Farrens and Khorana (42). These results suggest that the lifetime of R* is prolonged in 0.08% Cymal-5 when compared with R* in 0.015% DM. Moreover, the rate of R* decay in 0.08% Cymal-5 was significantly reduced in the presence of Gt, with less than 20% of the R* being converted to opsin and free all-trans-retinal. Taken together, these findings clearly show that R* in 0.08% Cymal-5 interacts with Gt to form a complex that triggers guanine nucleotide exchange, thereby facilitating analysis of R*/G-protein interactions under suitable NMR experimental conditions.

Monitoring Light-activated R*-catalyzed Guanine Nucleotide Exchange in the 15N-ChiT-reconstituted Heterotrimer by Solution NMR—To simulate the reaction scheme of the R*/G-protein interaction, which leads to the formation of Gα(GTP) (Fig. 1B), 15N-ChiT-reconstituted heterotrimer was first dialyzed into Buffer A to which 0.08% (w/v) Cymal-5 had been added. One-dimensional 15N-filtered 1H and two-dimensional HSQC spectra, which allow selective monitoring of amide and side chain 1H, 15N resonance signals from 15N-ChiT (~150 μM), were then acquired (data not shown) and compared with spectra of the heterotrimer in Buffer A alone. Comparisons of the spectra showed no differences in either amide or side chain chemical shifts or line widths, indicating that addition of 0.08% Cymal-5 does not affect either the conformation or stability of 15N-ChiT. The nonhydrolyzable GTP analog, GTPγS (~175 μM), was then added to the 15N-ChiT-reconstituted heterotrimer sample (~150 μM), and one-dimensional 1H- and 15N-filtered 1H spectra were acquired (Fig. 3, A and B (red traces)). The 15N-filtered 1H spectrum again showed no change in the 15N-ChiT signals with respect to the spectrum acquired for the 15N-ChiT-reconstituted heterotrimer, indicating that the added GTPγS remains free in solution and is not exchanged with bound GDP. The one-dimensional 1H spectra further support this conclusion as two new relatively sharp signals are observed to appear after addition of GTPγS, indicated by arrows in Fig. 3B, that are attributed to the anemic (~5.8 ppm) and aromatic H8 protons (~8.1 ppm) of the added GTPγS free in solution.

In contrast, addition of purified, dark state, rhodopsin that had been dialyzed into Buffer A containing 0.08% Cymal-5, to 15N-ChiT-reconstituted heterotrimer (~150 μM) in Buffer A with 0.08% (w/v) Cymal-5 and GTPγS (~175 μM) to a final concentration of 15 μM, resulted in an approximately 50% reduction in the observed 15N-ChiT amide and side chain proton signal intensity (Fig. 3A, light blue trace). This reduction in the signals was more pronounced than could be explained by simple dilution of the sample (addition of 75 μl of rhodopsin to 250 μl of reconstituted heterotrimer) and suggests that the heterotrimer may interact with Cymal-5-solubilized dark state rhodopsin. As would be expected, however, the one-dimensional 1H spectrum (Fig. 3B, light blue trace) showed no apparent changes in the signals of the free GTPγS, indicating that Cymal-5-solubilized dark state rhodopsin does not stimulate guanine nucleotide exchange.

After exposure of the NMR sample mixture (15N-ChiT-GtpγS-GDP/Mg²⁺ + GTPγS + solubilized rhodopsin) to light (~495 nm) for 1 min, there was an increase in peak intensities (Fig. 3A, green trace) that continued until a final state was reached (Fig. 3A, dark blue trace), suggesting formation of GTPγS/Mg²⁺-bound ChiT dissociated from R* and GtpγS. It should be noted based on the measured rate for R*-catalyzed guanine nucleotide exchange (Fig. 2B) that the exchange reaction in the NMR sample would also be rapidly completed within minutes after illumination. Most surprisingly, changes in the intensity of the ChIT signals detected in the one-dimensional NMR experiments (Fig. 3A) were still observed after several hours and even after changes in the intensity of the proton signals from the added GTPγS were observed to be complete. Given this significantly slower “rate” observed for the change in the intensity of the ChIT signals in the NMR experiment, the associated change in the state of ChIT does not appear to be associated with R*-catalyzed guanine nucleotide exchange. In addition, because Cymal-5-solubilized rhodopsin was added to the NMR sample in sub-stoichiometric amounts relative to Gt, it also appears that R* catalytically interacts with the heterotrimer under our NMR experimental conditions. We therefore came to the conclusion that the slow rate observed in the NMR experiment reflects neither guanine nucleotide exchange nor release of the exchanged heterotrimer from R*, but rather is a measure of the slow dissociation of Ga(GTPγS) from Gβγ. In this respect, the observed increase in the intensity of the ChIT signal is
consistent with ChiT assuming a faster correlation time, which results in a narrowing of the NMR line widths, as would be expected upon heterotrimer dissociation. Over the time course of subunit dissociation, the observed NMR signals would be contributed from a smaller protein with a faster correlation time, i.e., ChiT (40 kDa) versus the heterotrimer (85 kDa). Alternatively, the observed changes in ChiT signal intensity could be the result of a slow alteration in the conformation of Gα after it dissociates from Gβγ. Although this is also a possible explanation, a lack of observed associated changes in 1H chemical shifts makes it less likely.

R*-catalyzed uptake and release of guanine nucleotides is also apparent from changes in the 1H signals (H8/H1) arising from added GTPγS after illumination. As shown in Fig. 3B, GTPγS uptake by ChiT in the R*-catalyzed reaction is directly observed as a reduction in the 1H signals arising from added GTPγS, as would be expected upon association of free nucleotide with a large protein or protein complex. Additional sharp signals are also observed in the final state spectra of the guanine nucleotide exchange reaction (Fig. 3B). Although these signals may correspond to free GDP, which would be expected to be released from the 15N-ChiT-reconstituted heterotrimer in the exchange reaction, their chemical shifts do not correspond to those measured for H1 and H8 proton signals of free GDP in Buffer A (data not shown). Further experimentation will therefore be necessary to establish a definitive assignment and ascertain the reason(s) for this apparent difference.
Given the observed slow rate of change in the ChiT spectrum after illumination, the NMR sample was allowed to remain overnight before further NMR analysis (total time ~16 h). Additional one-dimensional $^{15}$N-filtered and $^1$H spectra of the NMR sample ($^{15}$N-ChiT-GTP$_y$GDP/Mg$^{2+}$ + GTP$_y$S + R) collected after this time period revealed no further changes in the spectra. Upon observation of no additional changes in the one-dimensional spectra, an HSQC was then acquired in situ to probe the conformation of GTP$_y$S/Mg$^{2+}$-bound ChiT. The HSQC spectrum reveals clear changes in the chemical shifts for the amide and side chain $^1$HN, $^{15}$N resonances when compared with the GDP/Mg$^{2+}$-bound form of ChiT (Fig. 4A). Most interestingly, the changes observed in the R*-generated GTP$_y$S/Mg$^{2+}$-bound form relative to the GDP/Mg$^{2+}$-bound form are similar, but not identical, to those observed upon formation of the GDP-AlF$_4$ /Mg$^{2+}$ adduct (Fig. 4B) and the heterotrimer (34, 35). For example, two of the three assigned cross-peaks for the tryptophan indoles (Fig. 4C, left panel) shift to the same position in these states. However, one tryptophan indole (Trp-207), located in the functionally important switch II region of G$_w^*$, appears to broaden beyond detection in the R*-generated GTP$_y$S/Mg$^{2+}$-bound state, and the carboxyl-terminal Phe-350 residue (Fig. 4C, right panel) is completely shifted into the "activated" conformation. In addition, and in contrast to the HSQC spectra acquired for the GDP/Mg$^{2+}$- and GDP-AlF$_4$/Mg$^{2+}$-bound states of $^{15}$N-ChiT and the GDP/Mg$^{2+}$-bound form of the $^{15}$N-ChiT-reconstituted heterotrimer, the spectrum of the R*-generated GTP$_y$S/Mg$^{2+}$-bound ChiT shows non-uniform line widths, with a number of resonances, like those associated with Trp-207 indole ring, appearing to be significantly broaden, and a second subset of resonances observed to be considerably sharper than on average. These NMR line width observations suggest that certain parts of the structure of ChiT are dynamic and exchanging between distinct conformations in the R*-generated GTP$_y$S/Mg$^{2+}$-bound state in a way that affects both the local and global structure.

Comparisons of GTP$_y$S/Mg$^{2+}$-bound $^{15}$N-ChiT Generated by R* with GTP$_y$S/Mg$^{2+}$-bound $^{15}$N-ChiT Generated Independently of R*-To dissect the relative contributions of changes in the conformation and dynamics of GTP$_y$S/Mg$^{2+}$-bound $^{15}$N-ChiT that are related to interaction with R* (and $G_{w^*}$) from those associated with guanine nucleotide exchange, GTP$_y$S/Mg$^{2+}$-bound $^{15}$N-ChiT was also generated in an "R*-independent" manner by directly reconstituting the "empty pocket" state of G$_w^*$ with GTP$_y$S. The HSQC spectrum of GTP$_y$S/Mg$^{2+}$-bound $^{15}$N-ChiT generated in this way reveals changes in the chemical shifts for the amide and side chain $^1$HN, $^{15}$N resonances that are again indicative of a shift in the protein conformation to an activated conformation (supplemental Fig. S1). As with R*-generated GTP$_y$S/Mg$^{2+}$-bound $^{15}$N-ChiT, changes observed in the R*-independent generated GTP$_y$S/Mg$^{2+}$-bound form relative to the GDP/Mg$^{2+}$-bound form are similar, but not identical, to those observed upon formation of the GDP-AlF$_4$/Mg$^{2+}$ adduct and the heterotrimer (34, 35). For example, two of the three assigned cross-peaks for the tryptophan indoles (Fig. 4D, left panel) shift to the same position in both the R* and R*-independent generated GTP$_y$S/Mg$^{2+}$-bound forms of $^{15}$N-ChiT. In contrast, Trp-207, which appears to broaden beyond detection in the R*-generated GTP$_y$S/Mg$^{2+}$-bound state, is found to be shifted to a unique downfield position, relative to the GDP-AlF$_4$/Mg$^{2+}$ adduct and heterotrimer states of ChiT, in the GTP$_y$S-bound state of ChiT generated independently of R*. Moreover, the carboxyl-terminal Phe-350 residue in this GTP$_y$S-bound state (Fig. 4D, right panel) is not observed to shift significantly to the activated position.

In contrast to R*-generated GTP$_y$S/Mg$^{2+}$-bound ChiT, the spectrum of GTP$_y$S/Mg$^{2+}$-bound ChiT generated independently of R* shows relatively uniform line widths, as observed for the GDP/Mg$^{2+}$- and GDP-AlF$_4$/Mg$^{2+}$-bound states of $^{15}$N-ChiT and the $^{15}$N-ChiT-reconstituted heterotrimer. This observation is consistent with the notion
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that the differential conformational dynamics within GTPyS/Mg$^{2+}$-bound ChiT is a direct result of interactions with $G_{\alpha}$ and $R^*$. 

Biochemical Analysis of a Complex between a Soluble Mimic of $R^*$ and $G_{\alpha}$.—Previously, we have designed, constructed, and expressed soluble cytoplasmic surface polypeptides of rhodopsin by grafting different combinations of the cytoplasmic loops of rhodopsin onto a thioredoxin scaffold. Biochemical and structural studies (38, 52) have shown that some of these rhodopsin cytoplasmic fragment/thioredoxin fusion proteins exhibit properties similar to those of $R^*$. In particular, the HPTRX/CDEF fusion protein, which contains tandemly linked segments from the CD (amino acids 132–154) and EF (amino acids 231–252) loops of rhodopsin (Fig. 5A), was found to exhibit constitutive activity toward $G_{\alpha}$. Specifically, HPTRX/CDEF displayed virtually identical kinetics of $G_{\alpha}$ activation when compared with light-activated ROS rhodopsin (38), suggesting that the CD and EF loops in the fusion protein can adopt a conformation that is a close approximation to that present in $R^*$. It was also shown that HPTRX/CDEF bound $G_{\alpha}$ (340–350) peptides (52) inducing structural changes in these peptides analogous to those observed previously in studies with native, light-activated rhodopsin in ROS membranes (28–30).

To characterize further the interaction between HPTRX/CDEF and $G_{\alpha}$, a fluorescence assay for $G_{\alpha}$-protein activation has been used to monitor guanine nucleotide exchange as a function of HPTRX/CDEF concentration. As shown in Fig. 5B, addition of sub-stoichiometric amounts of HPTRX/CDEF relative to $G_{\alpha}$ elicits only partial GTPyS uptake. Subsequent additions of HPTRX/CDEF result in increased levels of guanine nucleotide exchange, ultimately saturating the response at a concentration nearly equal to that of $G_{\alpha}$. These findings suggest that HPTRX/CDEF, in contrast to light-activated ROS rhodopsin, lacks the ability to catalytically interact with $G_{\alpha}$ and raised the possibility that these proteins may form a stable complex. To obtain biochemical evidence for such a stable interaction, an equimolar mixture of HPTRX/CDEF and ChiT-reconstituted heterotrimer was incubated in the presence of GTPyS and then immunoprecipitated with an anti-$G_{\alpha}$ antibody followed by protein G-Sepharose. The bound proteins were analyzed by SDS-PAGE and immunoblotting using an antibody mixture containing the anti-rhodopsin antibody K42-41L (37), which recognizes the EF loop region of rhodopsin, and anti-$G_{\alpha}$ antibodies (presumably $G_{\alpha}$) to co-precipitate and support the hypothesis that the non-catalytic nature of HPTRX/CDEF is likely because of stable complex formation between HPTRX/CDEF and the GTPyS/Mg$^{2+}$-bound heterotrimer.

NMR Analysis of a “Trapped” $R^*$+$G_{\alpha}$/$G_{\beta}$-GTP Complex.—The results of our earlier work with HPTRX/CDEF (38, 52) combined with the results of the $G_{\alpha}$ activation and immunoprecipitation assays (Fig. 5, B and C) prompted us to attempt to apply NMR methods to follow HPTRX/CDEF-stimulated guanine nucleotide exchange in $^{15}$N-ChiT-reconsti-

**FIGURE 5.** Binding of $G_{\alpha}$ to HPTRX/CDEF, a soluble mimic of $R^*$. A schematic two-dimensional representation based on the crystal structure of rhodopsin (Protein Data Bank code 1HZX) for the amino acid sequence corresponding to the second (CD) and third (EF) cytoplasmic loop regions of bovine rhodopsin that were inserted between Pro-34 and Cys-35 in a mutant form of thioredoxin (HPTRX) to generate a soluble mimic of $R^*$. A Gly/Pro linker is present between the CD and EF loops, and between these loops and the thioredoxin scaffold (represented by zigzag lines). The gray cylinders show the positions of the TM helices in rhodopsin. B, activation of $G_{\alpha}$ by HPTRX/CDEF. The assay mixture (100 µl) initially contained 500 nM $G_{\alpha}$ and 750 nM GTPyS. After obtaining a stable baseline, the reaction was initiated by the addition of 200 nM HPTRX/CDEF and followed for 1000 s. A second aliquot of HPTRX/CDEF was then added bringing the concentration to 400 nM, and the reaction was followed for another 1000 s. This same procedure was repeated until saturation of the fluorescence response (an additional two times). The fluorescence measurements, which allow the rate of GTPyS uptake by $G_{\alpha}$, to be determined by monitoring the increase in intrinsic fluorescence of Trp-207, suggest that HPTRX/CDEF does not catalytically interact with $G_{\alpha}$. Representative data from two independent determinations are shown. C, immunoprecipitation of the HPTRX/CDEF-$G_{\alpha}^{\gamma}$-$G_{\beta}^{\gamma}$ complex. HPTRX/CDEF (lane 1) was detected with the anti-rhodopsin K42-41L antibody; ChiT (lane 2) was detected with an anti-$G_{\alpha}$ antibody, and $G_{\alpha}$ (lane 3) isolated from a mixture ($G_{\alpha}$, $G_{\beta}$, $G_{\gamma}$), which was incubated with a mixture of HPTRX/CDEF was detected with an anti-$G_{\alpha}$ antibody. The HPTRX/CDEF-$G_{\alpha}$-$G_{\beta}$-$G_{\gamma}$-GTPyS/Mg$^{2+}$-exchanged complex (lane 4) was immunoprecipitated with the anti-$G_{\alpha}$ antibody followed by protein G-Sepharose, and the individual components detected with a mixture of K42-41L, anti-$G_{\alpha}$, and anti-$G_{\beta}$ antibodies as described under “Experimental Procedures.” Note that the intensities of the signals in each of the lanes most likely reflect differences in the affinities of the antibodies used to detect the various components as equimolar amounts of protein were analyzed. The positions of molecular mass standards are shown on the left.
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In summary, the present results show that solution NMR methods can be used to track the cycle of guanine nucleotide exchange in an isotope-labeled $G_{\alpha}$-reconstituted heterotrimer that is triggered by light activation of detergent-solubilized rhodopsin. Moreover, these same methods can be used to track guanine nucleotide exchange in an isotope-labeled $G_{\alpha}$-reconstituted heterotrimer stimulated by a soluble mimic of $R^{*}$, which remains bound forming a trapped, stable intermediate complex. Overall, our study demonstrates how NMR approaches can allow simultaneous detection of both local and global changes in $G_{\alpha}$, associated with $R^{*}$-stimulated guanine nucleotide exchange. The observations made using the soluble mimic of $R^{*}$ also indicate that GDP/GTP exchange can be uncoupled from the process of heterotrimer release and dissociation, suggesting that distinct $R^{*}$-induced changes in $G_{\alpha}$ may facilitate these events.

DISCUSSION

The mechanism(s) by which $R^{*}$ catalyzes rapid and tightly regulated guanine nucleotide exchange by heterotrimeric G-proteins is not fully

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understood. We are applying solution NMR methods to probe the structural basis for the propagation of signals from R* to the G-protein, with the specific goal of developing more robust models for changes in Gα that accompany signal transfer from R*. For our studies, we are using the well characterized rhodopsin/transducin interaction of the vertebrate visual system, as it serves as the paradigm for understanding activated GPCR/G-protein interactions (2, 3). Moreover, knowledge of the biochemical steps in this reaction (Fig. 1B), combined with the availability of crystal structures, provides a solid starting foundation on which to build our understanding of the structural changes in both rhodopsin and Gα that accompany complex formation and R*-catalyzed guanine nucleotide exchange.

We have shown previously that a full-length isotope-labeled Gα chimera (ChiT) can be prepared in milligram amounts and that the expressed protein shares comparable properties to native Gα (34). Furthermore, ChiT can be reconstituted with Gαγδ to form functional heterotrimers that are amenable to analysis using solution NMR methods (35). These studies have not only provided new insights into the role of

![Comparison of HSQC spectra acquired for the 15N-ChiT-reconstituted heterotrimer at various steps of the reaction pathway. A, overlay of the two-dimensional 15N-HSQC spectrum of 15N-ChiT in the GDP/Mg2+/bound form of the heterotrimer (blue) and the HPTTRX/CDEF-GuCo/GTPγS/Mg2+/bound complex (red). The assigned 1HN, 15N cross-peaks for the three Trp indoles (W127, W207, and W254) are shown in box 1, and those for the carboxyl-terminal Phe-350 are shown in box 2. Differences in the conformations of ChiT in these different heterotrimer states are manifested in a number of changes in the chemical shifts of the 1HN, 15N cross-peaks and suggest that ChiT is in a distinct state that appears to represent heterotrimer for which GDP has been exchanged for GTPγS, and HPTTRX/CDEF remains bound. B, overlay of the two-dimensional 15N-HSQC spectrum of the R*-generated GTPγS/Mg2+/-bound form of 15N-ChiT (blue) and the HPTTRX/CDEF-GuCo/GTPγS/Mg2+/bound complex (red). The assigned 1HN, 15N cross-peaks for the three Trp indoles (W127, W207, and W254) are shown in box 1, and those for the carboxyl-terminal Phe-350 are shown in box 2. Although the conformations of ChiT in these states are manifested in a number of similarities in the chemical shifts for the 1HN, 15N cross-peaks, apparent differences are evident and again suggest two distinct states for these GTPγS/Mg2+/-bound forms of ChiT. C, expansion of the Trp indole (left panel) and Phe-350 (right panel) resonance regions of the HSQC spectra of the GDP/Mg2+/bound form of the 15N-ChiT-reconstituted heterotrimer (black), GDP-AlF4-/Mg2+/-bound 15N-ChiT (red), and the 15N-ChiT-reconstituted HPTTRX/CDEF-GuCo/GTPγS/Mg2+/bound complex (blue). Assignments for the 1HN, 15N cross-peaks of the Trp indoles and Phe-350 are indicated. Note that as seen for the R*-generated GTPγS/Mg2+/bound form of ChiT, the carboxyl-terminal Phe-350 amide 1HN, 15N cross-peaks is observed to completely shift to the activated position in the HPTTRX/CDEF-GuCo/GTPγS/Mg2+/bound form of 15N-ChiT, although the Trp-207 1HN, 15N indole cross-peak is broadened and shifted to a position close to that observed for a minor conformational form of Trp-207 (denoted by an asterisk) in both the GDP/Mg2+/-bound recombinant heterotrimer and the GDP-AlF4-/Mg2+/-bound form of 15N-ChiT. All spectra were acquired at pH 7.5 and 30 °C using a Bruker 600 MHz NMR Cryoprobe system as described under “Experimental Procedures.”
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G\(_\alpha\) in remodeling the conformation of \(G_\alpha\) to facilitate R\(^*\) binding and GDP/GTP exchange, but these studies formed the basis for pursuing further studies focused on R\(^*\)-induced changes in \(G_\alpha\) structure. As such, we have set out in this study to demonstrate a systematic NMR approach for tracking the complete cycle of guanine nucleotide exchange in a \(^{15}\)N-ChiT-reconstituted heterotrimer using light-activated, detergent-solubilized rhodopsin and a previously characterized soluble mimic of R\(^*\).

R\(^*\)-mediated Guanine Nucleotide Exchange in the ChiT-reconstituted Heterotrimer—A primary concern from the outset of these experiments was to identify a suitable detergent that would support the formation of metarhodopsin II or R\(^*\), the rhodopsin signaling photointermediate, and also facilitate R\(^*\)/G-protein interactions that trigger guanine nucleotide exchange at concentrations below the CMC of the detergent. Cymal-5 appeared to effectively serve this purpose as rhodopsin samples prepared in this detergent, like those in the commonly used DM, showed the characteristic ground state chromophore (\(\lambda_{max} \approx 500\) nm) that was converted to R\(^*\) upon photolysis (Fig. 2A). Moreover, initial rate analysis of the GDP/GTP\(^\gamma\)S exchange reaction of \(G_\alpha\) catalyzed by Cymal-5-purified ROS rhodopsin revealed virtually indistinguishable kinetics of activation from that observed for DM-purified ROS rhodopsin (Fig. 2B).

At equilibrium, however, the amount of GTP\(^\gamma\)S exchanged was significantly higher for R\(^*\) in Cymal-5 when compared with R\(^*\) in DM. This latter finding agrees with the observation that the lifetime of Cymal-5-purified R\(^*\) is prolonged when compared with R\(^*\) in DM, and could be stabilized further in the presence of \(G_\alpha\) (Fig. 2C). Collectively, these findings show that Cymal-5-solubilized and -purified ROS rhodopsin exhibits functional properties that are comparable, and even more desirable, with those of rhodopsin in DM.

The tracking of R\(^*\)-induced changes in the structure of \(G_\alpha\) over the course of R\(^*\)/G-protein interactions and correlating these changes with the uptake of GTP\(^\gamma\)S could be achieved using a series of one-dimensional NMR spectra (Figs. 3 and 6). These spectra, which could be acquired in a relatively quick fashion (<5 min), allowed us to observe time-dependent shifting and changes in signal intensity of protein amide side chain-associated \(^1\)HN, \(^{15}\)N resonances and guanine nucleotide-associated \(^1\)H resonances, over the course of the light-activated rhodopsin-catalyzed nucleotide exchange reaction (Fig. 3). The uptake of GTP\(^\gamma\)S in the reaction could be monitored directly (Fig. 3B), and the conformation of the resulting product of the reaction, dissociated GTP\(^\gamma\)S/Mg\(^2+\)-bound \(^{15}\)N-ChiT, could be analyzed in situ using NMR methods and compared with other functional states of ChiT previously generated and analyzed using similar NMR approaches (Fig. 4). It is worth noting that the addition of dark state rhodopsin to the NMR sample containing the \(^{15}\)N-ChiT-reconstituted heterotrimer, and GTP\(^\gamma\)S resulted in a significant reduction in the observed \(^{15}\)N-ChiT amide and side chain proton signal intensity (Fig. 3A, light blue trace). This reduction in the signals, which was more pronounced than could be explained by dilution of the sample, suggests \(G_\alpha\) associates with the dark state of rhodopsin and is consistent with the results of plasmon-waveguide resonance experiments (53, 54).

The apparent rate of change in ChiT signal intensity under our NMR experimental conditions suggests that heterotrimer dissociation proceeds with very slow kinetics. It has been fairly well established that \(G_{\alpha_{D}}\) dissociates in the presence of GTP (55), although recent \textit{in vitro} studies suggest that certain other \(G_\alpha\) subtypes may not dissociate from G\(_{\alpha_{D}}\) following R\(^*\)-catalyzed guanine nucleotide exchange (56). Based on the differences in experimental variables and conditions used in studies examining heterotrimer dissociation (55, 57, 58), the measured rate of subunit dissociation may vary widely depending on the presence or concentrations of detergents, membranes, guanine nucleotides, activating compounds (AlF\(_4\)), as well as receptors and downstream binding proteins/effectors.

As with ROS rhodopsin, the HPTRX/CDEF-stimulated guanine nucleotide exchange reaction could be monitored using one-dimensional NMR methods and showed GTP\(^\gamma\)S uptake as well as distinct signal changes in the amide resonances of \(^{15}\)N-ChiT associated with guanine nucleotide exchange. However, in contrast to the observation of the release of GTP\(^\gamma\)S/Mg\(^2+\)-bound \(^{15}\)N-ChiT in the native R\(^*\)-catalyzed reaction, the NMR data collected for the HPTRX/CDEF-stimulated guanine nucleotide exchange reaction did not show similar evidence of heterotrimer dissociation from this soluble mimic of R\(^*\) and subsequent release of activated \(^{15}\)N-ChiT from G\(_{\alpha_{D}}\) (Fig. 6). These observations, in combination with the results of \(G_\alpha\) activation and immunoprecipitation assays (Fig. 5, B and C), suggest that this GTP\(^\gamma\)S/Mg\(^2+\)-bound form of ChiT persists as part of a nondissociated heterotrimer to which HPTRX/CDEF remains bound. Such a complex is analogous to the R\(^*\)-\(G_{\alpha_{D}}\)-GTP intermediate of the R\(^*/\)G\(_i\) reaction pathway (Fig. 1B, step 4) and affords the opportunity to study the structure of \(G_\alpha\) in this R\(^*\)-bound state. Although it is not currently known as to why HPTRX/CDEF fails to dissociate from the GTP\(^\gamma\)S/Mg\(^2+\)-exchanged heterotrimer, it is possible that additional, key amino acids not present in this soluble mimic of R\(^*\), such as those found in the H8 region of rhodopsin, or a conformation not adopted by the grafted CD and EF loops of rhodopsin in the thioredoxin scaffold (38), are required to initiate heterotrimer release and dissociation.

Changes in the Carboxyl Terminus of \(G_\alpha\) Accompanying R\(^*\) Interactions—From the available G-protein crystal structures, particularly that for the heterotrimer (Fig. 1A), and the results of several biochemical experiments focused on R\(^*\)/G-protein interactions, a number of different proposals (32, 59, 60, 62) have been put forth to explain how structural changes in the receptor-interacting regions of the G-protein may lead to GDP release and GTP uptake. Each of the proposals suggests mechanisms that involve long range global changes in the structure of \(G_{\alpha_{D}}\) upon interaction with R\(^*\) to account for the fact that the guanine nucleotide-binding site in \(G_\alpha\) is located ~30 Å from the proposed R\(^*\)-interacting surfaces (see Fig. 1A), the so-called “action at a distance” hypothesis of heterotrimeric G-protein activation. Although the details of each of the proposed mechanisms varies considerably, a common element in all of them is a central role for the extreme carboxyl terminus of \(G_\alpha\) in functioning as a recognition element in R\(^*\)/G-protein interactions. Evidence in support of its involvement has been provided by experiments showing that ADP-ribosylation of Cys-347 on \(G_\alpha\) by pertussis toxin uncouples \(G_\alpha\) from R\(^*\) (16, 17), mutational studies of \(G_\alpha\) that have shown amino acid residues in the extreme carboxyl terminus are essential for R\(^*\)-\(G_\alpha\) binding and R\(^*\)-catalyzed activation (64 – 66), synthetic peptide studies with various \(G_\alpha\) (340 – 350) peptides that mimic the conformational effect of G\(_i\), by stabilizing R\(^*\) and/or competitively blocking R\(^*/\)G\(_i\) interactions (18, 32, 67 – 72), and chemical cross-linking of R\(^*\) to \(G_\alpha\) followed by mass spectrometry of the labeled \(G_\alpha\) peptide(s) (73). A potential route of communication between R\(^*\) and the guanine nucleotide pocket involves alterations in the \(\beta_6/\alpha_{helix}\) that are transmitted from the carboxyl terminus via the \(\alpha_{helix}\) helix (74, 75). Mutations in the \(\alpha_{helix}\) helix have been shown to dramatically increase basal guanine nucleotide exchange rates and reduce R\(^*\)-catalyzed activation rates. Furthermore, a mutation in the \(\beta_6/\alpha_{helix}\) loop has been shown to mimic the action of R\(^*\) by causing rapid guanine nucleotide exchange (76, 77).

In this study, chemical shift perturbation of Phe-350 resonances observed to be associated with the interaction of light-activated rhodopsin and \(^{15}\)N-ChiT-reconstituted heterotrimer indicate that the confor-
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FIGURE 8. A schematic representation of the proposed conformational changes in G<sub>a</sub> coupled to R*-catalyzed guanine nucleotide exchange. The model has been extended from a previous study examining changes in G<sub>a</sub>, accompanying heterotrimer formation (35). The GTase and helical domains of G<sub>a</sub>, are shown in different shades of green; G<sub>a</sub> is shown in yellow, and G<sub>b</sub> in blue. The region representing the guanine nucleotide binding pocket in G<sub>a</sub> is shown in blue in the ‘ground’ state, and in red or orange (see below) in the ‘activated’ states. GDP and GTP are shown in salmon and purple, respectively. Upon association of G<sub>a</sub>(GDP) with G<sub>b</sub>, G<sub>a</sub> undergoes structural changes in the R* interacting amino- and carboxyl-terminal regions, as well as the switch regions surrounding the guanine nucleotide binding pocket, and suggest that although G<sub>a</sub> binding changes the G<sub>a</sub> structure to a ‘preactivated’ form, it displays at least two conformational states for the carboxyl terminus. These changes in the G<sub>a</sub> structure may both potentiate R* interactions and pre-organize the guanine nucleotide binding pocket. In the ‘empty pocket’ state following R* catalyzed release of GDP, pre-organization of the guanine nucleotide binding pocket would facilitate GTase binding to form G<sub>a</sub>(GTP)<sub>G</sub>, which ultimately dissociates into G<sub>a</sub>(GTP) and G<sub>b</sub>. The results of this NMR study suggest further changes in the carboxyl-terminal and switch II regions of G<sub>a</sub>, upon formation and dissociation of the R*<sub>G</sub>(GTP)<sub>G</sub> complex. In particular, the carboxyl terminus appears to adopt a fully ‘activated’ state conformation, although switch II, as well as other regions of the G<sub>a</sub>(GTP) structure, appear to become more dynamic upon heterotrimer dissociation. In this schematic, these changes in released G<sub>a</sub>(GTP) are highlighted through different green shading for the GTase domain that also has an altered conformation for the carboxyl terminus and lacks an ordered amino terminus, and the orange color for the guanine nucleotide binding pocket. Such changes in the structure of the amino-terminal region of G<sub>a</sub> are consistent with observations from the crystal structures of the heterotrimer (26, 27) and fluorescent and site-directed spin labeling experiments (31).

mation of the carboxyl terminus of G<sub>a</sub>, completely shifts to an activated form in the GTP<sub>S</sub>/Mg<sup>2+</sup>-bound state, relative to the “ground” state conformation observed for GDP/Mg<sup>2+</sup>-bound ChiT. In comparison, the conformation of the carboxyl terminus in GDP/Mg<sup>2+</sup>-bound ChiT in the presence of AlF<sub>4</sub>− (Fig. 4, A and B) and in the reconstituted heterotrimer shows evidence of a slow exchange equilibrium between ground and activated conformations, with each state roughly equally populated in the AlF<sub>4</sub>− state and shifted more toward the activated conformation in the heterotrimer. Interestingly, the carboxyl terminus of ChiT in the HPTRX/CDEF<sub>G</sub><sub>a</sub><sub>G</sub><sub>TPS</sub>/Mg<sup>2+</sup>-exchanged complex also appears to adopt a fully activated conformational form (Fig. 7A). In contrast to our observation of a discrete shift in the conformation of the carboxyl terminus of ChiT from a ground to an activated state as follows by shifting of the amide 1H, 15N cross-peak of Phe-350, solution NMR studies utilizing a segmental isotope-labeled G<sub>a</sub> prepared through expressed protein ligation techniques have shown that certain side chains in the carboxyl-terminal region of the GDP/Mg<sup>2+</sup>-bound form of G<sub>a</sub> lose intensity upon AlF<sub>4</sub>− adduct formation (78), suggesting an ordering of these side chains. Although a change in conformation of the carboxyl terminus of G<sub>a</sub> upon AlF<sub>4</sub>− activation has also been shown by monitoring a fluorescent reporter group attached at position Cys-347 of G<sub>a</sub> (61), the reason for the apparent differences in the backbone and side chain dynamics of the carboxyl terminus in the GDP-AlF<sub>4</sub>−/Mg<sup>2+</sup>-bound state is unclear and will require further NMR experiments to be resolved.

Changes in the Switch II Region of G<sub>a</sub> Accompanying R* Interactions—
In contrast to the observation of a similar behavior for the carboxyl terminus in these two GTP<sub>S</sub>/Mg<sup>2+</sup>-bound forms of ChiT, differences are observed for the behavior of resonances associated with the indole ring of Trp-207, which is located in the functionally important switch II region of G<sub>a</sub>. In the R* -generated GTP<sub>S</sub>/Mg<sup>2+</sup>-bound form of ChiT, the Trp-207 indole resonances appear to be broadened beyond detection, although resonances assigned to Trp-207, while also exchange broadened, are observed in a new chemical shift position in the spectrum of the HPTRX/CDEF<sub>G</sub><sub>a</sub><sub>G</sub><sub>TPS</sub>/Mg<sup>2+</sup>-exchanged complex (Fig. 7B), relative to the position observed previously for the major conformations of this residue in other states of ChiT (34, 35). Interestingly, however, the position of the cross-peak observed for Trp-207 in the HPTRX/CDEF<sub>G</sub><sub>a</sub><sub>G</sub><sub>TPS</sub>/Mg<sup>2+</sup>-exchanged complex is close to the chemical shift position observed for a minor conformational form of the Trp-207 indole (Fig. 7C, denoted by an asterisk) in the spectra of both the GDP/Mg<sup>2+</sup>-bound 15N-ChiT-reconstituted heterotrimer and the GDP-AlF<sub>4</sub>−/Mg<sup>2+</sup>-bound form of 15N-ChiT. Although the functional significance of the conformation represented by this downfield-shifted Trp-207 indole cross-peak is not clear, it may represent an intermediate, dynamic conformation of the switch II region that is populated by interaction of the heterotrimer with R*.

Comparison of the NMR Results with the Crystal Structures—The 2.2 Å crystal structure of the GTP<sub>S</sub>/Mg<sup>2+</sup>-bound form of G<sub>a</sub> (22) does not provide any indication that the switch II region and/or other portions of G<sub>a</sub> would display a range of conformational dynamics. However, the GTP<sub>S</sub>/Mg<sup>2+</sup>-bound G<sub>a</sub> that was crystallized lacked residues 1–25 in the amino terminus, which were removed prior to crystallization by proteolysis. Because the G<sub>a</sub> chimera used in our solution NMR experiments contained the full G<sub>a</sub> amino- and carboxyl-terminal sequences, it is likely that the dynamics observed for the GTP<sub>S</sub>/Mg<sup>2+</sup>-bound form of ChiT is a consequence of the presence of these additional residues. In particular, the extreme amino terminus likely adopts a dynamically disordered form after G<sub>a</sub>(GTP) is released from the heterotrimer (31), which may result in fast exchange dynamics locally, as well as cause more global intermediate to slow exchange dynamic effects in G<sub>a</sub>. In this respect, it is interesting to note that the GTP<sub>S</sub>/Mg<sup>2+</sup>-bound form of ChiT generated independent of R* did not display a similar dynamic character, nor was the amide cross-peak of Phe-350 in this...
GTP\textsuperscript{\textgamma}-bound state observed to shift completely to the activated position as seen with R\textsuperscript{\gamma}. These results further support the idea that it is specific changes in the conformation of the carboxyl and amino termini of ChiT associated with heterotrimer reconstitution and subsequent interaction of the heterotrimer with R\textsuperscript{\gamma} that result in the differences in the conformational dynamics observed for these GTP\gammaS/Mg\textsuperscript{\textgamma\texttwo} forms of ChiT.

**Guanine Nucleotide Exchange Can Be Uncoupled from G\textsubscript{\textalpha} Dissociation**—Given that the carboxyl terminus and switch II regions of G\textsubscript{\textalpha} have already been observed using NMR methods to adopt a preactivated conformation that may facilitate R\textsuperscript{\gamma} binding and subsequent GDP/GTP exchange in the heterotrimer (35), it would seem reasonable that further structural transitions in these two regions of G\textsubscript{\textalpha} would be associated with R\textsuperscript{\gamma}-catalyzed GDP release and GTP uptake. Such structural coupling between distant portions of G\textsubscript{\textalpha} is the basis for the proposed action at a distance hypothesis, which as mentioned above has been considered in most structure-based mechanisms of R\textsuperscript{\gamma}-catalyzed guanine nucleotide exchange. However, the observation of a trapped intermediate GDP/GTP exchanged heterotrimer state in complex with the soluble mimic of R\textsuperscript{\gamma} (Fig. 7) suggests that guanine nucleotide exchange is not sufficient for heterotrimer release from R\textsuperscript{\gamma} and subsequent dissociation of activated G\textsubscript{\textalpha} from G\textsubscript{\textgammaS}. In particular, the observation that the switch II region of ChiT is in an activated conformation in the exchanged, complexed heterotrimer, together with the previous observation that the switch II region of ChiT already adopts a preactivated conformation in the GDP/Mg\textsuperscript{\textgamma\texttwo}-bound state observed to shift completely to the activated [empty] complex, should provide additional information about the conformational changes in G\textsubscript{\textalpha} accompanying R\textsuperscript{\gamma}/G-protein interactions.

**Functional Implications of R\textsuperscript{\gamma}-induced Changes in G\textsubscript{\textalpha}**—We have developed a working model based on our previous NMR observations that also incorporates other fundamental results focused on elucidating changes in the structure of G\textsubscript{\textalpha} that accompany heterotrimer formation and R\textsuperscript{\gamma} interactions (35). Based on the findings reported here, and elsewhere (31), we have extended this model (Fig. 8) to highlight the additional observed perturbations in the conformation of the receptor interacting amino- and carboxyl-terminal regions as well as switch II that arise upon formation and dissociation of the R\textsuperscript{\gamma}G\textalpha\textgammaS-GTP complex (Fig. 1B, steps 4 and 5). Specifically, the carboxyl terminus, which displays at least two conformational states in the heterotrimer, is as noted above found exclusively in an activated state in both the R\textsuperscript{\gamma}G\textalpha\textgammaS-GTP complex (the HPTRX/CDEF-G\textalpha\textgammaS-GTP\gammaS/Mg\textsuperscript{\textgamma\texttwo} complex) and in G\textsubscript{\textgammaS} (GTP) (the GTP\gammaS/Mg\textsuperscript{\textgamma\texttwo}-bound form of ChiT), although Trp-207 is detected in only the R\textsuperscript{\gamma}G\textalpha\textgammaS-GTP complex, suggesting an altered conformation for switch II in G\textsubscript{\textgammaS} (GTP). This latter apparent change in the conformational dynamics of G\textsubscript{\textalpha} could be a consequence of the R\textsuperscript{\gamma}-induced changes in the structure of the amino- and carboxyl-terminal regions of G\textsubscript{\textalpha} and/or subsequent guanine nucleotide exchange and dissociation of G\textsubscript{\textgammaS}. In either case, the increase in the apparent conformational flexibility of G\textsubscript{\textalpha} and switch II in particular, may have important functional ramifications for both interaction of G\textsubscript{\textalpha} with downstream effectors, as well as GTP hydrolysis.

**Conclusions**—We have demonstrated previously the ability to express and isolate milligram quantities of an isotope-labeled G\textsubscript{\textalpha} chimera ([\textsuperscript{15}N]-ChiT) that can be reconstituted with G\textsubscript{\textgammaS} to form functional heterotrimers (34, 35). We have now shown that [\textsuperscript{15}N]-ChiT-reconstituted heterotrimer forms functional complexes under NMR experimental conditions with light-activated, detergent-solubilized rhodopsin and a soluble mimic of R\textsuperscript{\gamma}, both of which trigger guanine nucleotide exchange. Collectively, the studies carried out to date demonstrate that solution NMR can be used to describe at atomic resolution changes in the structure and dynamics of G\textsubscript{\textalpha} that accompany heterotrimer formation and signal transfer from R\textsuperscript{\gamma} to the G-protein. A key aspect of our approach is the ability to generate and interrogate trapped R\textsuperscript{\gamma}-bound conformations of G\textsubscript{\textalpha}, which have so far proven refractory to analysis by other high resolution structural methods. Overall, our work demonstrates that new insights into the structure of G\textsubscript{\textalpha} can be gained from these methods, which will augment our understanding of the structural mechanism(s) underlying signal transduction that are currently based on available high resolution, static crystal structures for G\textsubscript{\textalpha} in various states. Future efforts aimed at NMR analysis of other R\textsuperscript{\gamma}-bound conformations of G\textsubscript{\textalpha}, in particular the R\textsuperscript{\gamma}G\textalpha[empty] complex, should provide additional information about the conformational changes in G\textsubscript{\textalpha} accompanying R\textsuperscript{\gamma}/G-protein interactions.

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Structural Changes in $G\alpha$ Accompanying GPCR Activation