Published in final edited form as:

J Struct Biol. 2013 October ; 184(1): 63–74. doi:10.1016/j.jsb.2013.02.016.

Linking receptor activation to changes in Sw I and II of G α proteins

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Abstract

G-protein coupled receptors catalyze nucleotide exchange on G proteins, which results in subunit dissociation and effector activation. In the recent β_2 AR-G α_s structure, portions of Switch I and II of G α are not fully elucidated. We paired fluorescence studies of receptor-G α_i interactions with the β_2 AR-G α_s and other G α_i structures to investigate changes in Switch I and II during receptor activation and GTP binding. The 2/3 loop containing Leu194 of G α_i is located between Switches I and II, in close proximity to IC2 of the receptor and the C-terminus of G α , thus providing an allosteric connection between these Switches and receptor activation. We compared the environment of residues in myristoylated G α_i proteins in the heterotrimer to that upon receptor activation and subsequent GTP binding. Upon receptor activation, residues in both Switch regions are less solvent-exposed, as compared to the heterotrimer. Upon GTP γ S binding, the environment of several residues in Switch I resemble the receptor-bound state, while Switch II residues display effects on their environment which are consistent with their role in GTP binding and G dissociation. The ability to merge available crystal structures with solution studies is a powerful tool to gain insight into conformational changes associated with receptor-mediated G α_i protein activation.

Keywords

G α_i ; G α_i ; Switch I/II; site-directed fluorescence; N-terminal myristoylation; 5 helix

Introduction

The structural determination of the β_2 AR in complex with G α_s paints a stunning picture of the receptor-G protein complex (Rasmussen et al. 2011). Upon activation, the carboxy terminus (CT) of the G protein β subunit inserts into a hydrophobic pocket created by the outward movement of TM6 on the receptor. The structure confirms the CT of G α as the major receptor interaction site on the G protein, previously implicated in many structural and functional studies (Hamm et al. 1988; Conklin et al. 1993; Dratz et al. 1993; Schwindinger et al. 1994; Natochin et al. 2000). For example, peptides derived from the C-terminus of G

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bind to activated receptors (Dratz et al. 1993; Liu et al. 1995; Brabazon et al. 2003; Scheerer et al. 2008) and compete with endogenous G proteins to turn off receptor signaling (Gilchrist et al. 2002). When a flexible 5-glycine linker is inserted into the CT of G_i, this perturbs both the rate and extent of receptor-mediated G protein activation (Natochin et al. 2001; Marin et al. 2002; Oldham et al. 2006). Interestingly, the β_2 AR-G_s structure also implicated several other receptor contact sites on the G protein, in addition to the CT (Rasmussen et al. 2011), confirming some earlier studies on receptor coupling in a variety of G subtypes. These include the 4/6 loop (Onrust et al. 1997; Bae et al. 1999; Cai et al. 2001) N helix (Hepler et al. 1996; Blahos et al. 2001; Itoh et al. 2001; Preininger et al. 2008; Hu et al. 2010), and the 2/3 loop (Ho et al. 2004). Switch I (Sw I) precedes the 2/3 loop, and is next to linker residues which connect the GTPase and helical domains. Switch II (Sw II) is located on the other side of 2/3 loop. Sw II participates in binding to G and effectors in a GTP-dependent and mutually exclusive manner. Thus, receptor-mediated conformational changes in Switch I and II have functional implications in the G protein cycle, from GDP release to GTP binding and G dissociation. This work examines the connection between receptor activation and changes in Sw I and II of myristoylated G_i proteins.

Receptors catalyze GDP release from the G protein, and stabilize the nucleotide-free G protein. In the absence of an activated receptor, G_i subunits exhibit a loss of structure in the nucleotide-free state (Tontson et al.; Thomas et al. 2012). Because properly folded G_i proteins are difficult to isolate in the nucleotide-free state, proteins with mutations in the C-terminal 5 helix have been used to mimic the nucleotide-free state of the G_i protein (Kapoor et al. 2009; Preininger et al. 2009). However, this approach reveals neither how the receptor prevents the unfolding of the nucleotide-free G_i protein, nor how this conformation relates to subsequent GTP binding. From comparison of GDP-bound and GTP S-bound G proteins, we know that GTP binding is accompanied by dramatic changes in Sw I and Sw II (Noel et al. 1993; Lambright et al. 1994; Sprang 1997), but the conformation of these regions in the receptor-bound complex is not completely known. This is because, in the β_2 AR-G_s structure, parts of Sw I are disordered, and the nanobody stabilizing the complex binds to a portion of Sw II (Rasmussen et al. 2011). In an effort to better understand changes in these regions, we use site-specific fluorescence to illuminate the environment of residues in Sw I and II during receptor activation and GTP binding at several distinct end states. First, we examined the environment of the residues in Sw I and II in the inactive heterotrimer, and then compared it to their environment in the receptor-bound, nucleotide-free state, and finally after addition of GTP S to the G protein-receptor complex. We combined our fluorescence results with structural features available from G_i and G_s crystal structures in a hybrid fashion to learn more about how the receptor stabilizes the nucleotide-free state of the G protein, and how this relates to subsequent GTP binding.

Materials and Methods

Protein expression, labeling and purification

Construction, expression, and purification G_i proteins were performed as described previously (Medkova et al. 2002; Preininger et al. 2003). Briefly, a parent G_i lacking solvent-exposed cysteines (HI), which was previously shown to have properties similar to wild type (Medkova et al. 2002), was generated with an expression vector encoding rat G_i, with the following amino acids substituted: (C66A-C214S-C305S-C325A-C351I), a hexahistidine tag inserted between amino acid residues M119 and T120 (Medkova et al. 2002; Van Eps et al. 2006). Wild-type G_i proteins for supplementary experiment contained native residues throughout, except for mutations as indicated in S. Fig. 1 and the hexahistidine tag used to facilitate purification. Mutations were introduced using the QuikChange system (Stratagene, La Jolla, CA); DNA sequencing confirmed all mutations. Myristoylated proteins were obtained by co-expression with N-myristoyl transferase (NMT,

with NMT vector generously provided by M. Linder, Washington University) and purified as detailed previously (Preininger et al. 2003). Coomassie staining of urea SDS PAGE gels (Mumby and Linder 1994) demonstrate G_i proteins are fully myristoylated (Preininger et al. 2003) when co-expressed with the NMT vector. Purified proteins were stored at -80°C in buffer containing 50 mM Tris, 100 mM NaCl, 2 mM MgCl₂, 10 μM GDP, pH 7.5, and 10% (v/v) glycerol. After purification, all proteins used in this study were greater than 90% pure, as estimated by Coomassie staining of SDS-PAGE gels. The ability of the purified proteins to undergo activation-dependent changes was ascertained with intrinsic tryptophan fluorescence (see below).

Preparation of rod outer segments (ROS) membranes containing bovine rhodopsin and G_i 1: urea-washed rod outer segment membranes (ROS) containing dark-adapted rhodopsin were prepared as previously described (Mazzoni et al. 1991) and stored protected from light at -80°C in buffer containing 10 mM MOPS, 200 mM NaCl, 2 mM MgCl₂, 1 mM dithiothreitol (DTT) and 100 μM phenylmethylsulfonyl fluoride (PMSF). G_i 1 (hereafter referred to as G_i) was purified from bovine retina as described previously (Mazzoni et al. 1991) and stored in a buffer containing 50 mM HEPES, 100 mM NaCl, 5 mM β -mercaptoethanol (BME), pH 7.5, with 10% (v/v) glycerol.

Protein Labeling: G_i HI proteins were labeled as described previously (Preininger et al. 2012) using a 10 molar excess of AlexaFluor⁵⁹⁴C₅-maleimide, (A1) (Invitrogen, WI), with a labeling time of 2 hours for Sw II proteins and 4 hours for Sw I proteins. Labeled proteins were purified via gel filtration HPLC, and fractions screened by intrinsic Trp fluorescence (or BD-GTP S binding, in the case of W211C G_i HI proteins, as described below) to ensure the functional integrity of the labeled proteins. Labeling efficiency was determined from comparison of Abs₅₈₀ to protein concentration, as determined by Bradford, and found to be between 0.25–0.4 mol_{label}/mol_{protein}, depending on the location of the residue and labeling time. The monodispersity and molecular weight of the monomeric, labeled proteins after HPLC gel filtration purification were confirmed through comparison of peak retention times and peak shape to a broad range molecular weight standard run on the same day as the purified samples (Bio-Rad, Hercules, CA). These labeled G_i Hexa I proteins retain the ability to undergo activation-dependent changes, bind G_q, and couple to rhodopsin in ROS membranes (Medkova et al. 2002; Preininger et al. 2008).

Fluorescence Assays

Intrinsic Trp fluorescence: purified G_i proteins demonstrated > 40% increase in intrinsic Trp emission upon AlF₄ activation, relative to emission in the GDP-bound state, as previously described (Mazzoni and Hamm 1993). Briefly, G_i proteins (200 nM) in buffer A consisting of 50 mM Tris, 100 mM NaCl, 1 mM MgCl₂, pH 7.5 containing 10 μM GDP were monitored by fluorescence, with $\lambda_{\text{ex}} = 290\text{ nm}$, $\lambda_{\text{em}} = 345\text{ nm}$, both before and after the addition of AlF₄ (10 mM NaF and 50 μM AlCl₃) using a Varian Cary Eclipse Fluorometer. For G_i proteins containing a W211C mutation, the ability of the proteins to undergo activation-dependent changes was verified using BD-GTP S Invitrogen (Madison, WI) as described previously (Hamm et al. 2009). Briefly, nucleotide exchange was measured as fold-increase in emission of 1 μM BODIPY-GTP S (BD-GTP S, $\lambda_{\text{ex}} = 490\text{ nm}$, $\lambda_{\text{em}} = 515\text{ nm}$) in buffer containing 50 mM Tris, 100 mM NaCl, 1 mM MgCl₂, 1 μM GDP, pH 7.5, at 21°C for 30 minutes after the addition of G_i subunits (200 nM) to the cuvette containing BD-GTP S. Tryptophan fluorescence of heterotrimeric G_i proteins in the absence of receptors: indicated G_i proteins (400 nM, unmyristoylated) were reconstituted with G_q (400 nM), and resulting heterotrimers were scanned with ex/em 280/340 both before and after activation with AlF₄ (Suppl. Fig. 1).

Receptor-mediated fluorescence changes: labeled G_i proteins were reconstituted with G at 4 °C prior to addition of excess dark ROS containing inactive rhodopsin, which was then transferred to a cuvette containing buffer A at a final concentration of 200 nM G_i protein, 250 nM G_i, and 2 μM dark-adapted rhodopsin. All manipulations involving dark rhodopsin were performed under dim red light to prevent its activation. The emission (dark spectra) from the cuvette containing dark-adapted ROS and A1-labeled subunits in complex with G_i was scanned between 590–750 nm with excitation at 580 nm at 21 °C using a Varian Cary Eclipse. After collecting dark spectrum, cuvettes were removed and subjected to a flash of light; light spectra were collected in duplicate 10 minutes after photo activation of rhodopsin. Finally, GTP S spectra were collected in duplicate 10 minutes after addition of GTP S (40 μM) to the light-activated sample in each cuvette. While duplicate readings were averaged for the light and GTP S spectra, the dark spectrum was collected only once prior to light activation in order to avoid spurious activation of rhodopsin. Spectra average of three independent experiments (± S.E.M.) (Preininger et al. 2008).

Receptor-mediated nucleotide exchange rate: the rate of receptor-catalyzed GDP/GTP S exchange was measured by monitoring the time-dependent increase in the Trp²¹¹ fluorescence (ex/em 290/340 nm) of G_i HI proteins reconstituted with G_i (200 nM each) in buffer A- (lacking GDP) in the presence of 100 nM rhodopsin (light activated) at 21 °C (Oldham et al. 2006). Nucleotide exchange was initiated by the addition of GTP S (10 μM). The data (average of 3 or more independent experiments) were normalized to the baseline (0%) and the fluorescence maximum (100%). The exchange rate was determined by fitting the data to an exponential association curve using Prism 4.0 (GraphPad Software).

Membrane binding assay

Rhodopsin binding assay: the ability of labeled G_i proteins to bind rhodopsin in urea-washed ROS membranes was determined by a reconstitution assay. A1-labeled G_i and G_i (5 μM each) were incubated together on ice for 30 minutes in buffer A- prior to adding light-activated rhodopsin (50 μM) to the reconstituted G protein sample. The samples were incubated with ROS for an additional 30 minutes, and an aliquot was further treated by addition of GTP S (40 μM). Following 30 minutes incubation on ice, the membranes in each sample were pelleted by centrifugation at 20,000 X *g* for 1 hour, and supernatants removed from pellets. The isolated supernatant and pellet fractions were boiled, resolved by SDS-PAGE, and visualized with Coomassie blue (Preininger et al. 2008).

Structural depictions and statistical analyses

Molecular graphics images were produced using the UCSF Chimera package (Sanner et al. 1999; Pettersen et al. 2004) and PyMOL Molecular Graphics System (DeLano 2002). Graphs and statistical analysis were performed using GraphPad Prism 4.0, GraphPad Software, San Diego California USA. Sequences were aligned with Clustal W using the human G_i sequences and accession codes as follows: G_{i1} P603096, G_{i1} P11488, G_{i2} Q03113, G_{i3} Q14344, G_{i4} P50148, G_{i5} P63092 (UniProtConsortium).

Results and Discussion

In order to examine changes within specific regions of the G_i subunit, we used a parent G_i protein lacking solvent-exposed cysteines as a background for introduction of Cys residues at sites of interest. The parent G_i protein, G_i HI, and its labeled derivatives, binds to G_i and couples to rhodopsin in ROS membranes (Medkova et al. 2002; Preininger et al. 2003; Preininger et al. 2008). We labeled cysteine residues with the thiol-reactive AlexaFluor (A1) probe, and examined changes in the environment of the probe. These were measured first in the heterotrimeric state, in the presence of inactive, membrane bound receptors. We then

compared the environment at each position to that in the receptor-bound, nucleotide-free, state, and finally to that in the GTP γ S-bound state. The isolation of the activated G protein-receptor complex was facilitated by use of a nucleotide-free buffer and G α_i proteins in their myristoylated form. After addition of GTP γ S to the receptor-bound complexes, the labeled G α_i proteins are released from receptors, and recovered in the soluble fraction (Preininger et al. 2008; Hamm et al. 2009). Throughout this study, this protocol was used to examine the environment of labeled residues during the G protein cycle. Because there is no crystal structure of a G α_i -receptor complex, or for any GDP-bound G α_s subunit, we have combined our solution studies of G α_i proteins with existing G α_i and G α_s structures, to help better understand conformational changes that accompany activation in Sw I and II.

A triad of residues forms a hydrophobic pocket at the receptor-G α interface

Structural determination of the β_2 AR-G α_s complex shows residues in the 2/3 loop in close proximity to the second intracellular loop (IC2) of the β_2 AR (Rasmussen et al. 2011). A conserved hydrophobic residue in IC2 (F139 in β_2 AR) occupies a position important for coupling of G α subunits to class A GPCRs (Chen et al.; Moro et al. 1993; Wacker et al. 2008). In the structure of the complex, the density reveals IC2 residue F139 in close proximity to residues homologous to G α_i L194 and G α_i F336 (Fig. 1A–B, G α_i numbering throughout)(Rasmussen et al. 2011), thus forming a triad of hydrophobic residues linking the receptor, the 2/3 loop and CT of G α (Fig 1A–B). To determine if receptor activation is communicated to changes in the 2/3 loop in G α_i proteins, we reconstituted fluorescently-labeled G α_i L194C protein (L194C-A1), with G $\beta_1\gamma_1$ (G $\beta\gamma$), and incubated the heterotrimer with dark adapted rhodopsin (Rho) from rod outer segment (ROS) membranes. We scanned the emission of the inactive G protein from 590–750 nm in the presence of dark-adapted Rho (Fig. 1C, black trace) using excitation wavelength 580 nm, which does not activate Rho (Imamoto et al. 2000; Preininger et al. 2008). We next subjected the sample to a flash of light to activate receptors and re-scanned the sample (Fig. 1C, blue trace). The increased emission intensity of the from the labeled G α_i L194 upon receptor activation indicated a less polar environment for the A1 probe, consistent with increased packing (Imamoto et al. 2000; Harikumar et al. 2002; Smith et al. 2004; Preininger et al. 2008) surrounding G α_i L194 in the receptor-bound, nucleotide-free state.

To determine if mutation of G α_i L194 to Cys perturbs receptor-mediated G protein activation, we measured the GDP-GTP γ S exchange rate in the presence of activated receptors. The rate of nucleotide exchange closely approximates the rate of GDP release, which is the rate-limiting step in G protein activation (Mukhopadhyay and Ross 1999). Mutation of G α_i Leu 194 to Cys significantly decreased the rate of receptor-mediated nucleotide exchange in the unlabeled protein (Fig. 1D, red), as compared to the parent protein containing the native Leu at position 194 (Fig. 1D, black). This is consistent with a role for G α_i 194L in receptor-mediated activation of G α_i proteins.

The receptor contributes F139 to the hydrophobic triad of residues pictured in Fig. 1A–B. This Phe, located in IC2 of the receptor, is conserved across class A GPCRs, and is located next to an invariant Pro (Wacker et al. 2008), P138 in β_2 AR. This Pro likely helps to position Y141 in the IC2 helix for interaction with D130 in the DRY motif. This interaction also likely helps position F139 on the opposite side of the IC2 helix for interaction with the G protein (Fig. 1B). Interestingly, a Leu to Ser mutation of the residue homologous to G α_s F139 in a G α_q -coupled class A GPCR, GPCR54, results in idiopathic hypogonadotropic hypogonadism (Wacker et al. 2008). Mutations of this residue to Asp or Glu even more dramatically impairs receptor-mediated G protein activation *in vitro* (Wacker et al. 2008), together underscoring the importance of a hydrophobic residue at this position for productive interaction with G α_q family proteins. A similar role in G α_i family proteins has yet to be established.

The receptor-mediated fluorescence changes we observed in G_i L194C-A1, and the decreased rate of receptor-mediated exchange in the L194C mutant, may be due to the interaction this residue makes with activated receptor, supporting a role for the 2/3 loop in receptor interaction. The L194C mutation may also disrupt the allosteric connection to F336 in the C-terminal 5 helix, which itself is directly linked to receptor activation. The hydrophobic nature of G_i L194 is conserved across G protein families (L, I or V), and F336 in the C-terminal 5 helix is absolutely conserved (Fig. 1E). While little is known about mutations of this residue in G_i proteins, mutation of the homologous Phe in the C-terminus of monomeric G protein Ras disrupts the 1-4 sheet network (Quilliam et al. 1995), accelerating the rate of nucleotide release. Likewise, a link between the 5 helix and 2/3 loop is implicated in receptor-mediated activation of G_q proteins (Ho et al. 2004). Thus, the allosteric network of interactions surrounding this hydrophobic triad of residues may play important roles in receptor-mediated nucleotide release. Since the 2/3 loop is located between Sw I and II (Fig. 1E), we next sought to determine if receptor activation was communicated to residues within Sw I.

Receptor-mediated changes in Sw I of G α proteins

Sw I connects the helical and GTPase domains of G proteins, thus it is often referred to as the Sw I linker. Sw I consists of residues in the 2 strand in the GTPase domain plus residues making up the 2/ F loop which connects it to the helical domain. In the 2AR-G_s structure, the Sw I residues that are not a part of the 2 strand are disordered and therefore not visible in the structure (Rasmussen et al. 2011). To learn more about how domain separation, which is mediated by the Sw I linker, affects residues adjacent to this disordered region, we measured changes in emission intensity of labeled residues in Sw I of G_i proteins before and after receptor activation (Fig. 2A, blue vs. black bars, as in 1C). The black bars (and black line) represent the maximal emission intensity of each respective residue in the inactive state, set to 1.0. These are set to 1.0 because the environment of each of the labeled residues in the heterotrimer is unique. Since the only difference between the dark and light samples is the flash of light used to activate receptors, the results shown in Fig. 2A reflect changes in the environment of each of the labeled as a result of receptor activation.

Residue V179C-A1 in the N-terminus of the Sw I linker, which is the first ordered residue flanking the disordered portion of the Sw I linker in the 2AR-G_s structure, displayed a relatively similar level of fluorescence both before and after light activation. This labeled protein, like all of the labeled proteins used in our study, retained the ability to undergo activation-dependent changes (see methods), thus the lack of environmental change reflects a relatively similar average polarity surrounding the probe before and after receptor activation. Farther up Sw I, nearer residues in the GTPase domain, we observed receptor-mediated enhancement in emission at all of the remaining positions tested (Fig. 2A), starting with G_i T182. These results are consistent with increased packing throughout the Sw I region upon receptor activation. These results also rule out unfolding of the 2 strand upon receptor activation, as this would expose these residues to solvent, which would decrease, not increase emission from the labeled residues. To better understand the structural basis for the changes we observed in Sw I (Fig. 2A), we compared the conformation of Sw I in the G_i heterotrimer to the conformation of the homologous residues that were well ordered in the 2AR-G_s structure.

Despite the fact that the individual G protein structures used for comparison are at higher resolution than that of the complex, the individual residues in the 2AR-G_s structure selected for structural comparisons with G_i proteins exhibited occupancy-weighted B factors which were within the average range for this structure (Kleywegt et al. 2004). This is a known metric used to judge the quality of crystal structures in the PDB (Brown and

Ramaswamy 2007), and it was used, along with evaluation of the density at each position, to select residues for structural comparisons in this study. In the G_i heterotrimer, I184 and V185 point in opposite directions, with I184 oriented towards the inter-subunit space between G_{α} and G_{β} (Fig. 2B, left panel, black). Upon receptor activation, residues homologous to G_{α} I184 and V185 (G_s I207 and F208), stack over one another (Fig. 2B, left panel, red, G_i numbering throughout, see Suppl. Table 1). The enhanced emission from G_{α} I184C-A1 is consistent with the stacking of I184 over V185 upon receptor activation in the G_i -receptor complex. In the inactive G_i heterotrimer, the α 2 strand is stabilized through hydrogen bonding with adjacent strands (1GP2, (Wall et al. 1995)). However, in the receptor-bound state, the hydrogen bonding at the N terminus of the α 2 is not present in the α 2AR- G_s structure (Rasmussen et al. 2011), effectively shortening the α 2 strand. The stacking of residues upstream of this region may help stabilize the folding of the α 2 strand during the process of domain separation. This domain separation is likely a result of the loss of contacts between the domains upon interaction of the G protein with activated receptors, which is accompanied by a wide distribution of distances between the domains (Van Eps et al. 2011) and a high degree of flexibility overall (Abdulaev et al. 2006).

How might changes communicated from receptor to the α 2 strand link to GDP release? While these experiments don't address this question, one potential mechanism may involve destabilization of bound Mg^{+2} , which is seen coordinated to GDP through its interactions with the conserved T181 in the crystal structure of G_{α} -GDP- Mg^{+2} (Coleman and Sprang 1998). A role for Mg^{+2} -mediated stabilization of GDP binding is implicated by the structure of G_{α} T329A. This mutant exhibits a loss of coordination between T181 in Sw I and Mg^{+2} (Kapoor et al. 2009), and displays an elevated rate of basal nucleotide exchange, similar to that mediated by activated receptors. This same study demonstrated that low Mg^{+2} concentrations alone are sufficient to elevate rates of nucleotide exchange in wild-type G_{α} proteins, again supporting the role of Sw I- Mg^{+2} interactions in maintaining high-affinity GDP binding. While it has long been known that Mg^{+2} is not required for GDP binding (Higashijima et al. 1987), it is not entirely unexpected that Mg^{+2} may promote GDP binding, given its role in GTP binding. Perturbation of Sw I's coordination with the Mg^{+2} -GDP binding site may help trigger nucleotide release in G_i proteins. This likely occurs in concert with disruption of the interface between the guanine ring and the base of the α 5 helix, such as in the T329A mutant (Kapoor et al. 2009). In the visual system, Mg^{+2} binding may reduce conformational dynamics of G_{α} that is normally associated with binding to activated receptors (Abdulaev et al. 2006). This NMR study demonstrated that, in the absence of activated receptors, the conformation of Mg^{+2} -bound, GDP-free G_{α} resembles that of G_{α} -GDP, supporting a role for Mg^{+2} in stabilizing the inactive conformation of the G_{α} protein.

Farther up the α 2 strand are G_{α} residues I184 and E186. These residues are absolutely conserved across G protein families, and mutations of these residues exert subtle effects on receptor-mediated nucleotide exchange (Oldham et al. 2007). Like I184C-A1, G_{α} E186C-A1 exhibited increased emission upon receptor-mediated activation (Fig. 2A). While residues corresponding to G_{α} E186 are themselves unaltered by activation (Fig. 2B, right panel), nearby residue homologous to G_{α} K197, G_s H220, is reoriented upon receptor activation. The density for G_s H220 indicates the presence of a hydrogen bond between its α -nitrogen and the nearby α -carboxyl of E209 (G_{α} E186). A similar reorientation of G_{α} K197 may contribute to the enhanced fluorescence we detected for G_{α} I186 in Fig. 2A. The enhanced fluorescence along the α 2 strand may stabilize the α -sheet structure at the core of the GTPase domain during receptor mediated domain separation, mediated by linker residues N-terminal to the α 2 strand. Since Sw II is located on the other side of the α 2/ α 3 loop that contacts receptor, we next sought to determine if residues in Sw II sensed receptor activation.

Receptor-mediated changes in Sw II of G α_i proteins

Because activation-dependent conformational changes in Sw II regulate binding to G and effectors in a mutually exclusive manner, and because of its location downstream of the 2/3 loop, we next examined receptor mediated changes in Sw II. Sw II encompasses the 2 helix which is specifically involved in binding to G and contains Trp²¹¹, a well-conserved reporter of conformational changes in G proteins (Mazzoni et al. 1993; Lan et al. 1998). We labeled residues throughout this region, and found that Lys209C-A1 displayed a robust increase in emission upon receptor activation (Fig. 3A, blue vs. black line). Likewise, S206C-A1 also displayed a large increase in emission (Fig. 3B). The receptor-mediated increases for S206C-A1 and K209C-A1 ruled out a solvent-exposed orientation for these residues, which would result in decreased, not increased emission. We compared these fluorescence results to the structure of the G $_i$ protein (Wall et al. 1995), and not to that of the activated complex, because of the extensive contacts that the nanobody makes with Sw II (Fig. 3C, right inset). In the G $_i$ structure, residues K209 and S206 of G $_i$ are on the face of the 2 helix that contacts G (Fig. 3C, center, heterotrimer in black, corresponding to black in bar graphs). The increased emission that we detected for these two residues is consistent with enhanced packing between residues in the N-terminus of the 2 helix and G.

Midway through the 2 helix lies the well-known Trp involved in activation-dependent changes in G proteins, W211 in G $_i$. Crystal structures of G $_i$ proteins activated by GTP S and AlF₄⁻ positions W211 in a hydrophobic groove bounded by the 2 and 3 helices (Coleman et al. 1994; Berghuis et al. 1996). We observed an increase in emission of G $_i$ W211C-A1, which indicated increased packing surrounding this labeled residue upon receptor activation. Nearby residues H213C-A1 and S214C-A1 exhibited similarly modest increases in emission upon receptor activation (Fig. 3B). In the G $_i$ structure, H213 and C214 are oriented towards the inter-subunit space between G and G subunits (Fig. 3C), directly over the central cavity of the G barrel (Wall et al. 1995; Lambright et al. 1996) (Fig. 3D). Taken together, the receptor-mediated increases in fluorescence throughout the 2 helix indicate a closely packed Sw II interface in the receptor-activated complex (Cherfils and Chabre 2003).

While an earlier spin-label study detected receptor-mediated changes at G $_i$ G217C (as well as at several positions in Sw I) (Oldham et al. 2007), the spin-labeled proteins did not detect receptor-mediated changes at other spin-labeled 2 helix residues (Van Eps et al. 2006), as might be expected upon binding to G. One reason for this may be that G $_i$ proteins used in the current study are myristoylated, which enhances the affinity of G for G (Linder et al. 1991), while the previous spin-label study used unmyristoylated proteins. Another reason may be that although the smaller spin label is optimal for detecting backbone level fluctuations, the somewhat larger A1 probe (MWt. 900) is better able to sense packing between subunits. Both the spin and fluorescent probe detected changes at G $_i$ G217. The 2AR-G $_s$ structure indicates that G $_i$ 217G is not likely in contact with the receptor, as the homologous G $_s$ residue (D240) is more than 17 Å away from the nearest receptor contact. However, the amide hydrogen of neighboring T242 in G $_s$ is hydrogen bonded to the backbone carbonyl of R42 in G $_s$, a residue homologous to G $_i$ K35 in the N terminus of G $_i$. This backbone interaction replaces a similar backbone interaction between amide nitrogen of K35 with the carbonyl of A220 in G $_i$ prior to receptor activation (Wall et al. 1995). The new backbone interaction between residues homologous to K35 and A219 in G $_i$ more closely links Sw II to the N terminus in the receptor-bound complex (Fig. 3E). Furthermore, in the receptor-bound complex, residue 32 of in the N-terminus of G $_s$ is linked to the receptor through a van der Waals contact with S143, which lies adjacent to the receptor's DRY motif. The interaction between the NT and receptor may propagate to a change in packing between G $_i$ K35 and A219 directly adjacent to Sw II, suggesting a conduit between activated receptors and Sw II of G $_i$ proteins. Consistent with this idea,

receptor activation results in conformational changes in Sw II (as well as the C-terminus) of G_i proteins (Ridge et al. 2006).

Changes in environment of Sw I residues upon receptor-mediated GTP γ S binding

To determine how GTP binding alters the environment in Sw I, we added GTP γ S to the activated G_i -receptor complexes and scanned the emission of the labeled proteins in the GTP γ S-bound state. Our experimental approach recapitulates the sequence of events that occur during the G protein cycle at three discrete endpoints: heterotrimer (measured in the presence of inactive receptors, Fig. 4A, basal) receptor-bound, active complex (Fig. 4A, blue bars) and the soluble, GTP γ S-bound state (Fig. 4A, green bars). Despite the ability of these proteins to undergo activation-dependent changes in Trp emission and partition to the soluble fraction on GTP γ S binding (see methods), the environment of several Sw I residues after GTP γ S binding mirrored their environment in the receptor-bound state (Fig. 4A, blue vs. green bars). Val 179C-A1 exhibited similar emission intensities in the inactive, receptor-bound and GTP γ S-bound states (Fig. 4A). This is consistent with the relatively solvent-exposed position for G_i V179 seen in both the heterotrimer and GTP γ S crystal structures, and its location adjacent to disordered residues in the receptor-bound G_s structure. The increase in emission intensity for T182C-A1 on binding GTP γ S is higher than basal, and is similar to its emission in the receptor-bound state (Fig. 4A). This suggests a well-packed environment in both the receptor- and GTP γ S-bound states at position 182. Because this position adjoins the disordered span of Sw I residues in the receptor-bound structure, we compared the environment of G_i T182 in the G_i -GTP γ S structure to the environment of this residue in the intact G_i protein. In the GTP γ S-bound G_i structure (Coleman et al. 1994), T182 is hydrogen bonded to the nearest side chain, E207. In the heterotrimer, T182 and E207 are nearly 17 Å apart, consistent with the relatively lower emission we detected for this labeled residue in the basal (heterotrimeric) state.

Likewise, we observed a similar environment of G_i I184C-A1 before and after GTP γ S binding (Fig. 4A). In the G_i structure, residue I184 is the first residue in the $\alpha 2$ strand. Like other A1-labeled G_i proteins, G_i I184C-A1 translocates to the soluble fraction on GTP γ S binding (Fig. 4B). To gain insight into the structural changes responsible for the high level of fluorescence for residue 184 upon GTP γ S binding, we compared the orientation of the homologous residue in the receptor-bound complex (G_s I207) to I184 in the G_i -GTP γ S structure. As shown in red in Fig. 4C (and 2B), the residue homologous to G_i I184 stacks over an adjacent residue in the receptor-bound complex. In the G_i -GTP γ S structure, this stacked orientation is replaced by a van der Waals contact between I184 and C214 (Fig. 4C, green), thus explaining the well-packed environment for both of these residues upon GTP γ S binding (Fig. 4A and Fig. 5A).

Farther up the $\alpha 2$ strand, G_i E186 exhibited a drop in emission upon GTP γ S binding (Fig. 4A, 4D), suggesting that GTP binding increases the solvent exposure at this position. In the G_i -GTP γ S structure, E186 does not interact with K197, but in the $\alpha 2$ AR- G_s structure, there is clear density supporting a salt bridge between the homologous residues, E209 and H220, prior to GTP γ S binding (Fig. 4E, green vs. red, respectively). This is consistent with the decrease in emission of G_i E186C-A1 upon GTP γ S binding. Finally, at the top of the $\alpha 2/3$ loop is L194, part of the hydrophobic triad pictured in Figs. 1A–B. This labeled residue maintained high emission intensity upon GTP γ S binding (Fig. 4A). This was unexpected, given the loss of interaction with the receptor, as well as loss of interaction with CT residue F336 upon GTP binding, which moves the CT out of the receptor binding pocket. However, these losses are compensated by a new interaction with residue V34 in the N/1 hinge upon G_i -GTP γ S binding (Fig. 4F, green, GTP γ S-bound vs. red, receptor-bound).

The interaction between N/1 hinge and the 2/3 loop is reflected in the high emission of G_i L194C-A1 in the GTP S-bound state. However, since this interaction is also present in the structure of the G_i heterotrimer, it is not likely to be the only cause of the fluorescence we observed. Further investigation revealed that the CT influences the environment of the hinge region. To examine the relationship between the CT and N/1 hinge region, which makes van der Waals contact with residue 194 in the 2/3 loop, we tested the ability of R to L mutation (R32L) in the hinge to mediate changes in a protein with a Trp mutation at the C terminus (L353W). We compared Trp emission in the inactive heterotrimers to Trp emission after activation with AlF₄. This circumvents the need for receptor-mediated GDP/GTP S exchange, as AlF₄ directly complexes with bound GDP, forming a transition state mimetic of GTP (Coleman et al. 1994). It also allows direct comparison of heterotrimer vs. AlF₄-activated states using Trp fluorescence. We found that while neither mutation alone had any effect on Trp fluorescence, the combination of these mutations significantly altered the activation-dependent change in Trp fluorescence (S. Fig. 1). This links the CT to the N/1 hinge region that contacts L194. While not conclusive, this indirectly supports an increased proximity to the CT upon GTP S binding (as compared to the basal state), which likely contributes to the relatively high fluorescence of G_i L194C-A1-GTP S.

We do not know the reason for the similarly high emission of Sw I residues in the receptor-bound and GTP S bound states. The enhanced packing of Sw I residues in the receptor-bound state may help stabilize the protein in the absence of nucleotide, given its location adjacent to the linker which mediates domain separation. Maintaining a solvent-restricted environment in the receptor-bound state may also be energetically favorable in terms of subsequent GTP binding, as it circumvents a desolvation penalty that would otherwise be associated with bringing GTP-Mg⁺² into a hydrated binding site. The maintenance of the environment from receptor-bound to GTP-bound state is consistent with the observed kinetics of GTP binding, which quickly follows the rate-limiting step of GDP release. Because the binding of GTP is sensed by Sw II, we next examined the environment of Sw II residues upon GTP S binding.

Changes in environment of Sw II residues upon receptor-mediated GTPyS binding

The activation-dependent reorganization of Sw II upon GTP S binding influences the residues in the 2 helix in a position-specific manner. When GTP S was added to the receptor-bound complexes, both G_i S206C-A1 and K209C-A1 maintained elevated levels of emission intensity (Fig. 5A). Comparing emission of these residues in the GTP S-bound state, where the structure is known, to emission in the receptor-bound state, where the environment of these residues is obscured by the nanobody, provides valuable insights into conformational changes associated with activation in the Sw II region.

Because the nanobody binds portions of Sw II, we used the structure of the G_i heterotrimer, PDB file 1GP2, to help visualize the Sw II and the G_i-G_i interface prior to GTP binding. In the G_i heterotrimer, residues S206 and K209 are more than 10 Å apart from each other (Fig. 5B–C, black). After GTP S binding, there is an activation-dependent increase in proximity between 206 and 209 (Fig. 5B–C, green) due to hydrogen bonding between the positively charged side chain of K209 and the backbone carbonyl of S206. The fluorescent probe on S206C provides a convenient reporter of this increased proximity. The enhanced emission from K209C-A1 upon GTP S binding suggests that this labeled residue senses the GTP-dependent reorganization of Sw II, which brings K209 into close proximity with S206 (Fig. 5B–C).

Farther up the Sw II 2 helix, we observed a modest increase in G_i W211C-A1 and S214C-A1 upon binding to GTP S, compared to basal (Fig. 5A, green bar vs. black line). Trp²¹¹, a well-known reporter of activational changes in G_i, is oriented towards the core of

the protein in both the heterotrimer and the GTP S-bound states (Fig. 5B–C). Note that the folding of W211C-A1 was confirmed using a fluorescently labeled GTP S analog, since mutation of the native Trp precludes use of the Trp activation assay (see methods). We used this approach since Bodipy-labeled GTP S exhibits a time-dependent increase in fluorescence on binding to G α that mirrors the GTP S-dependent increase in W211 in the unlabeled parent protein (Preininger et al. 2012). Although we selected mainly solvent-exposed positions for site-directed labeling in this study, W211 is oriented towards the core of the protein, and activation by GTP S increases the packing of this residue within the 2-3 binding pocket. While the 5-carbon spacer linking the thiol to the fluorescent moiety facilitates flexibility of the probe, the 2-3 pocket may not accommodate a fluorophore as well as other, more solvent exposed positions. Nevertheless, the activation-dependent increase in fluorescence we observed in G α W211C-A1 indicates that the increased packing of this labeled residue against the core of the protein is likely maintained through GTP S binding. As mentioned previously in connection with Sw I residue 184, G α S214C-A1 shows an enhanced emission in the GTP S-bound state (Fig. 5A). Again, this is consistent with the interaction between C214 and I184 seen in the crystal structure of G α GTP S (Fig. 4C, green). On the other hand, positions H213 and G217 in G α both exhibit a decrease in emission intensity upon GTP S binding (Fig. 5A, green bars). In the G α GTP S structure, H213 is solvent-exposed upon dissociation from G β (Fig. 5C, green). The drop in emission that we observe at position 213 suggests that the contacts with G β are reduced or eliminated upon GTP S binding. Likewise, the decreased emission exhibited by G217C-A1 upon binding GTP S (Fig. 5A) indicates an increase in solvent exposure for G217, consistent with a loss of packing between neighboring G α G219 and the N/1 hinge (Fig. 3E).

It is important to note that, unlike GDP-bound G α s, for which there are no high resolution structures, there are several structures for GTP S-bound G α s. Comparison of G α sGTP S from 1AZT (Sunahara et al. 1997) revealed that the G α s residues are similar in their respective orientations as the homologous residues in G α i pictured in Figs. 4 and 5 in green. In the case of G α s H220, homologous to G α i K197 (Fig. 4e, green), H220 of G α s is hydrogen bonded to a solvent phosphate ion in the G α sGTP S structure. Despite this artifact, the distance between G α s 209 and 220 (G α i 186 and 197, respectively) increases after GTP S binding, qualitatively consistent with the drop in emission we observe in the homologous residue in G α i (E186) upon GTP S binding. While the 2AR-G α s structure is the first of an activated receptor in complex with a G protein, the use of stabilizing proteins may alter native conformation, especially in regions of direct contact. However, site-specific labeling has its own drawbacks. Backbone conformational changes may result in detection of changes in environment not sensed by a native residue, due to the larger radius of detection of the fluorescent probe. Furthermore, since the emission represents the average environment of the probe, fluorescence may not be particularly informative in highly flexible and disordered regions of the protein. Nevertheless, these site-specific labeling experiments provide additional information regarding the environment of regions which are not completely elucidated in the structure. Together with the high resolution structures of individual G proteins, this work highlights the sequential changes in Switch regions of G that occur upon receptor activation and GTP binding (Fig. 6).

The observed changes in Sw II upon GTP binding can be understood within the context of the canonical view of the G protein cycle, including GTP-dependent G α – dissociation (schematic, Fig. 6A and model, 6B). Receptor activation results in the nucleotide-free, empty pocket G β subunit, in complex with G γ and receptor, which stabilizes the high affinity state of the receptor. Subsequent GTP binding to G α results in its dissociation from both receptors and G β , each competent to activate their respective downstream effectors. Hydrolysis of bound GTP returns the G α to the GDP-bound state, leading to G

reassociation, with the G protein poised for a new round of activation. While several studies have indicated that G_αGTP and G_{βγ} subunits may remain tethered (Frank et al. 2005; Ridge et al. 2006), our results do not support an interaction between Sw II of G_α and G_{βγ} upon GTP binding to G_α. However, further studies with specifically labeled G_α and G_{βγ} subunits may reveal tethering through other regions of G_α, or a possible tethering through distinct scaffold proteins. Importantly, while G_{αs} and G_{αi} are not likely to be identical in many regions of their structure, the conformations of the Sw I and II residues depicted in the G_{αi}GTP-S structure (Coleman et al. 1994) were qualitatively reproduced in the G_{αs}GTP-S structure (Sunahara et al. 1997). The similarities between Sw I and II in the G_{αs} and G_{αi}GTP-S-bound structures likely reflect the conserved nature of these Switch regions in all G proteins.

4.0 Conclusion

The current study follows the environment of Sw I and II residues upon receptor activation and subsequent GTP binding during the G protein cycle. Receptor activation is allosterically communicated to Sw I and Sw II through a hydrophobic triad of residues. These originate from a residue homologous to F139 in IC2 of the β_2 AR, and G_{αi} residues 194 in the 2/3 loop and F336 in the C-terminal α 5 helix. Of all the residues in Sw I and Sw II examined, only G_{αi}L194 is close enough to make direct receptor contact, according to homologous residues in the β_2 AR-G_{αs} complex. Another contributor to the hydrophobic triad is F336 of G_{αi}. Receptor activation and translocation of the C-terminal α 5 helix into the receptor binding pocket brings F336 into proximity with L194 of G_{αi} and F139 of the IC2 loop of the receptor. The residue G_{αi}L194 that is linked to receptor activation is located in the 2/3 loop that is sandwiched between Sw I and Sw II. Our fluorescence results suggest that receptor activation is accompanied by enhanced packing within the 2 strand of Sw I. This may serve to stabilize Sw I during separation of helical and GTPase domains, mediated by residues N-terminal to the 2 strand. Consistent with structures of isolated G proteins in the activated state, GTP-S binding stabilizes the relative orientation between the domains. In our experiments, the environment of Sw I residues observed in the receptor-activated state was maintained upon subsequent GTP-S binding. Downstream from the 2/3 loop, labeled residues in Sw II display increased packing upon receptor activation, followed by position-specific changes upon GTP-S binding. The GTP-dependent changes in Sw II reflect reorganization of this region that occurs upon GTP binding and G_α dissociation. The receptor-mediated changes in Sw I and Sw II may help preserve the folding of the protein in the nucleotide-free state, stabilizing the protein for fast, efficient GTP binding to the receptor-bound G protein. The current study combines results from solution studies with insights from crystal structures to reveal new details associated with the sequence of conformational changes in Sw I and Sw II that occur during receptor activation and subsequent GTP binding to G_{αi} proteins.

Supplementary Material

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Acknowledgments

Research reported here was supported by National Institutes of Health Grants EY006062 and GM095633 (to H.E.H.). The authors gratefully acknowledge helpful discussions with Drs. T. M. Iverson and V. V. Gurevich and, and thank G. Liao and S. Meier for technical assistance. We also thank R. A. Tracy for proofreading the manuscript.

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Fig. 1. Receptor-mediated changes in the 2/3 loop

A. Structure of the γ 2AR-G_s complex from PDB file 3SN6 (Rasmussen et al. 2011). G_s is shown in blue, receptor in orange, G_i in olive, G_o gold, antibody fragments in grey. Sw I is shown in pink, Sw II in yellow. Red circle encompasses a hydrophobic triad of residues in close proximity to receptor's DRY motif. **B.** Close up view of A, hydrophobic triad residues in red, and residues linked to conserved DRY motif in γ 2AR shown in green, indicated distances separating hydrophobic side chains shown with dotted lines. Residue D130 is part of the DRY motif (γ 2AR 130–132). G_i numbered according to homologous residue in G_s (see Suppl. Table 1 for listing of homologous residues in G_s vs. G_i, γ 2AR vs. Rho). **C.** Emission of labeled G_i protein in complex with G_s is scanned from 585–735 nm, with excitation at 580 nm, in the presence of either dark adapted (black) or light activated (blue) rhodopsin. **D.** Mutation of L194 significantly decreases the rate of receptor-mediated nucleotide exchange. Black, parent G_i protein; red, L194C. Exchange is initiated by addition of excess GTP_s to unlabeled G_i proteins (in complex with G_s) in the presence of light activated rhodopsin. Inset: quantitation of receptor mediated nucleotide exchange from at least 3 independent experiments ($\pm$ S.E.M.). **E.** Sequences in Sw I and Sw II of

indicated G_i proteins were aligned using UniProt (UniProtConsortium), with G_s sequences followed by the UniProt identifiers: G_{i1} P603096, G_t P11488, G_{i2} Q03113, G_{i3} Q14344, G_q P50148, G_s P63092). Secondary structure is depicted above sequences, and residue 194 from hydrophobic triad is outlined in red.

2A

2B

2C

Fig. 2. Receptor-mediated changes in Sw I

A. Maximal emission intensity as a result of receptor-mediated changes in the indicated labeled residues, blue bars, as compared to environment of the same labeled residue in the inactive, basal state (black bars). Data are the average of 2–3 independent experiments (+ S.E.M.). **B.** Residues I184 and V185 in G_i heterotrimer shown in black (PDB file 1GP2 (Wall et al. 1995)) overlaid with GTPase domain of activated β_2 AR-Gs complex (PDB 3SN6), homologous residues in activated complex shown in red, G_i numbering throughout (see Suppl. Table 1). **C.** Overview of β_2 AR-Gs active complex from PDB file 3SN6. G_i Sw I residues which were mutated to Cys and labeled are shown in red, inset is overview of complex. Red dashed line represents region of Sw I which is disordered in β_2 AR-Gs

structure. NB refers to nanobody (antibody fragment) used to stabilize the activated complex, shown in light grey.

Fig. 3. Receptor-mediated changes in Sw II of G proteins. **A.** Representative spectra of light dependent changes in Sw II, shown for K209C-A1, quantitated for all Sw II residues examined in B. **B.** Maximal emission intensity as a result of receptor-mediated changes in the indicated labeled residues, blue bars, as compared to environment of the same labeled residue in the inactive, basal state (black bars). Data are the average of 3 or more independent experiments (+ S.E.M.). **C.** Left inset: overview G – interface in G (inactive GDP-bound heterotrimer, PDB file 1GP2). G shown in black, G in dark grey. Center: close up of the G – interface in 1GP2, hydrogen bonds shown with dashed lines between indicated residues. Right inset: close up view of specific interactions between Sw II residues and antibody fragment (nanobody, NB) shown in light grey (PDB file 3SN6). **D.**

Slab view of indicated residues in the α_2 helix of Sw II, viewed from on top of the G barrel, with the remainder of G not shown for clarity. **E.** Close up view of interactions between backbone of residue 219 with the neighboring α_1 strand, taken from the α_2 AR-Gs structure (PDB file 3SN6).

Fig. 4. Changes in Sw II upon receptor-mediated GTP S binding

A. Maximal emission intensity upon receptor-mediated GTP S binding (green bars) to the light activated complexes (blue bars). Black line indicates maximal emission intensity in the inactive heterotrimer (basal). Data are the average of 3 or more independent experiments ($\pm$ S.E.M.). **B.** G α labeled at residue 186 is membrane bound in the light activated state (LS, soluble fraction from light activation; LP, light, pellet fraction; GS, soluble fraction after addition of GTP S to the light activated sample; GP, pellet fraction after GTP S addition). **C.** Residues 184 and 214 interact in the GTP S-bound G α (1GIA (Berghuis et al. 1996)), shown in green, as compared to the homologous residues in the α 2AR-Gs structure (side chains in red). **D.** Representative trace of emission from labeled residue 186 before and after GTP S binding. Receptor-bound, blue trace; GTP S-bound, green trace. **E.** Residues homologous to G α 197 and 186 form a salt bridge in the α 2AR-Gs activated complex (PDB 3SN6, side chains in red), in contrast to the position of these residues in the G α 1GTP S structure (PDB 1GIA), shown in green. **F.** Overlay of G α 1GTP S and G α s from α 2AR-Gs (PDB 1GIA and 3SN6, respectively), highlighting two residues in the hydrophobic triad, Gi 194 and 336, and a residue in the N terminus of G α i, V34, which makes a new hydrophobic contact with L194 upon GTP S binding.

Fig. 5. Sw II changes due to receptor-mediated GTP S binding

A. Maximal emission intensity upon receptor-mediated GTP S binding (green bars) to the light activated complexes (blue bars). Black line indicates maximal emission intensity in the inactive heterotrimer (basal). Data are the average of 3 or more independent experiments (+ S.E.M.). **B.** Overview of GTP S bound G α_i (green, PDB file 1GIA) compared to G α_i heterotrimer (black, PDB file 1GP2). **C.** Close up view of the isolated G α_i interface shown in B.

Fig. 6. Schematic and model

Top panel represents experimental protocol, corresponding to discrete steps in the G protein cycle, modeled in the bottom panel. Inactive receptor, red; active receptor, orange; $G\alpha$ - GDP, black and grey; nucleotide-free $G\alpha$ ($G\alpha_{(empty)}$), blue, with $G\alpha$ in black and grey; $G\alpha$ - GTP, green; GTP, red; GDP, black.