Mapping allosteric connections from the receptor to the nucleotide-binding pocket of heterotrimeric G proteins

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Heterotrimeric G proteins function as molecular relays that mediate signal transduction from heptahelical receptors in the cell membrane to intracellular effector proteins. Crystallographic studies have demonstrated that guanine nucleotide exchange on the $G\alpha$ subunit causes specific conformational changes in three key “switch” regions of the protein, which regulate binding to $G\beta\gamma$ subunits, receptors, and effector proteins. In the present study, nitroxide side chains were introduced at sites within the switch I region of $G\alpha i$ to explore the structure and dynamics of this region throughout the G protein cycle. EPR spectra obtained for each of the $G\alpha(\text{GDP})$, $G\alpha(\text{GDP})\beta\gamma$ heterotrimer and $G\alpha(\text{GTP}\gamma\text{S})$ conformations are consistent with the local environment observed in the corresponding crystal structures. Binding of the heterotrimer to activated rhodopsin to form the nucleotide-free (empty) complex, for which there is no crystal structure, causes prominent changes relative to the heterotrimer in the structure of switch I and contiguous sequences. The data identify a putative pathway of allosteric changes triggered by receptor binding and, together with previously published data, suggest elements of a mechanism for receptor-catalyzed nucleotide exchange.

G protein receptor complex | site-directed spin-labeling | switch I | visual signal transduction

Heterotrimeric G protein α subunits function in the cell as molecular switch proteins, cycling between an inactive GDP-bound heterotrimeric conformation and an active GTP-bound conformation. Heptahelical receptors in the cell membrane activate G proteins by catalyzing GTP for GDP exchange on $G\alpha$, leading to the activation of downstream effector proteins by $G\alpha(\text{GTP})$ and $G\beta\gamma$. The signal is terminated upon the hydrolysis of GTP to GDP by $G\alpha$ and its reassociation with $G\beta\gamma$ (1).

Nucleotide-dependent conformational changes in $G\alpha$ have been identified by x-ray crystallography. A comparison of the GDP-bound (2) and GTP γ S-bound (3) structures of the transducin α subunit ($G\alpha t$) identified three segments of the protein that undergo rearrangement upon activation, named switches I–III. Similarly, the corresponding structures of $G\alpha i$ demonstrate a GTP γ S-dependent conformational change in switch I, whereas switches II and III, which are disordered in the GDP-bound structure (4), become ordered upon GTP γ S binding (Fig. 1A) (5). Switches I and II interact directly with the γ -phosphate of GTP and form part of the $G\beta\gamma$ -interacting surface in the heterotrimer (Fig. 1B) (6, 7). Switch II is an important effector-binding site on $G\alpha$ (8–11), and a recent site-directed spin-labeling (SDSL) study showed that nucleotide exchange results in an increase in switch II dynamics, an event that may play a role in effector recognition/binding (12). Switch I plays a more critical role in binding RGS (regulators of G protein signaling) proteins (11, 13). Clearly, these switch regions form the basis of the conformational switching mechanism in $G\alpha$ by sensing the identity of the bound nucleotide and regulating G protein interactions with other molecules.

$G\alpha$ subunits of heterotrimeric G proteins consist of two domains: a GTPase domain, which resembles monomeric G proteins, such as

Ras, and a helical domain that buries the guanine nucleotide-binding pocket in the core of the protein. Switch I is a loop that forms one of the two linkers between these domains by connecting the α F-helix of the helical domain to the β 2-strand of the GTPase domain (Fig. 1A). Upon exchange of GDP for GTP γ S, switch I is drawn toward the nucleotide-binding pocket, establishing stabilizing interactions with Mg^{2+} and the γ -phosphate (2, 4).

Switch I is located at sites of crystal contact in both the GDP- and GTP γ S-bound structures for all $G\alpha$ structures determined thus far, suggesting the possibility that the conformation of this region may be different in solution. Moreover, crystal structures necessarily hide the important role that protein dynamics can play in regulating protein function. Because switch I plays a crucial role in the structural changes leading to G protein activation, it is important to investigate the native structure and dynamics of this region in solution. Toward this end, SDSL was used to monitor changes in the switch I sequence of $G\alpha i$ through each transition leading to G protein activation using activated rhodopsin (R^*) as the receptor. For each of eight spin-labeled mutants containing the nitroxide side chain R1 (12), EPR spectra were recorded for four states: $G\alpha i(\text{GDP})$, $G\alpha i(\text{GDP})\beta\gamma$, $R^*\cdot G\alpha i$ (0) $\beta\gamma$ (the “empty complex”), and $G\alpha i(\text{GTP}\gamma\text{S})$. The spectra are characteristic of the local protein structure (14, 15) and encode information on backbone dynamics (16). Thus, the spectra provide information on the solution structure and functional dynamics of the switch I region at each step in G protein activation, enabling comparisons with the crystal structures where available. Most importantly, the data provide insight into conformational changes involving switch I that accompany the formation of the receptor-bound empty complex.

Results

Characterization of Spin-Labeled Mutants. Sites selected for the introduction of R1 in the switch I sequence (177–187) were residues 179, 180, and 182 in the switch I loop and residues 184, 186, and 187 in the β 2-strand. In addition, R1 was introduced at residue 171 in a contiguous sequence of the helical domain on the α F-helix and at residue 50 on the adjacent α 1-helix. Fig. 1A shows the position of these residues in the $G\alpha i$ subunit. Both $C\alpha$ and $C\beta$ carbons are shown as spheres to illustrate the direction that the native side chain projects in the structure. Except for buried residues 50 and 187, these sites are on the protein surface. Importantly, none of the side

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Abbreviations: PDB, Protein Data Bank; P-loop, phosphate-binding loop; R^* , activated rhodopsin; ROS, rod outer segment; SDSL, site-directed spin-labeling.

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Fig. 1. Models of *Gai* and *Gai* $\beta\gamma$ crystal structures showing the spin-labeled sites. (A) Overlay of *Gai*(GDP) [Protein Data Bank (PDB) ID code 1BOF] and *Gai*(GTP γ S) (PDB ID code 1GIA) structures identifying the switch sequences (red in GDP and green in GTP γ S) and sites where R1 was introduced with orange spheres (C α and C β ; GTP γ S-bound structure only). Spheres at C β show the projection of the native side chain within the structure. The GTP γ S ligand is shown as a space-filling model in blue. Switches II (SwII) and III (SwIII) are not resolved (disordered) in the *Gai*(GDP) structure and are not shown. Homologous sequences are resolved in the *Gai*(GDP) crystal structure and show conformational changes upon nucleotide exchange. The helical domain and P-loop (P) are indicated in yellow and pink, respectively. (B) Ribbon model of *Gai*(GDP) $\beta\gamma$ (PDB ID code 1GP2) showing positions within *Gai* (gray) where R1 was introduced. The switch I segment is green, and G β and G γ are purple and cyan, respectively. (C) Surface representation of the heterotrimer showing stick models of R1 at sites 179, 180, 182, and 186 in *Gai* that lie in the cleft between the G α and G β subunits. The contributions of switch I and P-loop residues to the surface are shown in green and pink, respectively, and the G β surface is purple. The nearest intramolecular contacts for 180R1 are residues in the P-loop. The I184 side chain makes direct contact with the G β subunit and is highlighted in red.

chains mutated in the switch I loop contribute directly to nucleotide binding.

All of the spin-labeled mutants formed stable R^{*}–G protein complexes, and all mutants demonstrate R^{*}–catalyzed increases in GTP γ S binding, except site 184R1 [supporting information (SI) Fig. 4]. Because residue 184 contributes directly to the G β -binding surface (Fig. 1C), this result is likely due to the reduced affinity of this spin-labeled mutant for G $\beta\gamma$ (see below) and the low protein concentrations used in the nucleotide exchange assays. Clearly, this mutant does bind G $\beta\gamma$ and R^{*} in a GTP γ S-dependent manner, as

demonstrated both by the rhodopsin binding assay (SI Fig. 4) and EPR spectral changes (see below), where higher protein concentrations are used. Interestingly, three of the spin-labeled mutants (residues 171, 182, and 184) had substantially faster rates of basal nucleotide exchange compared with the cysteine-depleted *Gai* base mutant (SI Fig. 4). After biochemical characterization, a series of EPR spectra were recorded for these mutants at each step in the G protein cycle from *Gai*(GDP) to *Gai*(GTP γ S) (Fig. 2).

In the present study, we qualitatively interpret the EPR spectra in terms of a generalized “mobility” of R1 on the nanosecond time scale as reflected by the peak-to-peak width of the central resonance lines (ΔH°) in the first derivative spectra and the splitting of hyperfine extrema where resolved (2Azz'; Fig. 2A) (17, 18). Increases in either of these quantities are correlated with decreases in mobility and vice versa. For each pair of spectra compared, the spectra are normalized to the same number of nitroxide spins, and increases and decreases in ΔH° are recognized by decreases and increases in spectral intensity.

***Gai*(GDP).** The spectra for *Gai*(GDP) mutants 179R1, 180R1, 182R1, 184R1, and 186R1 of switch I all have dominant components corresponding to fast ($\tau \approx 2$ ns) motion of R1 (Fig. 2A, black traces), and, with the exception of 179R1, the motion is essentially isotropic. The crystal structure predicts that the R1 side chain at each of these sites projects into solution (Fig. 1A), consistent with the highly mobile nitroxide observed by EPR. If the loop structure containing residues 180 and 182 and the edge strand of the β -sheet containing residues 184 and 186 were rigid, the spectra would be expected to reflect an ordered anisotropic motion (19–21) similar to that for R1 on rigid helices (22). Thus, the essentially isotropic motion of R1 at these sites suggests a flexible backbone in solution (16). Although the switch I sequence is at a lattice contact in the crystal structure of *Gai*(GDP) (4), the thermal B factors for C α are relatively high through the sequence. Indeed, residue 184 has one of the highest B factors in the structure. Thus, the EPR spectra are generally compatible with expectations based on the crystal structure and emphasize the flexibility of the backbone throughout the 179–186 sequence in solution.

The remaining sites, 50R1, 171R1, and 187R1, exhibit complex, multicomponent spectra reflecting both immobilized (i) and mobile (m) states of R1 (Fig. 2A, arrows) (14, 15). Again, this finding is consistent with the location of these sites in the structure; both 50R1 and 187R1 project into the same interior space in the protein fold (Fig. 1B) and are largely immobilized by tertiary interactions (14, 15). Residue 171R1 is the most solvent-exposed of these three residues and has the largest fraction of a mobile component but still forms immobilizing interactions with the α 1-helix across the inter-domain cleft (Fig. 1A).

Heterotrimer Formation. Switch I contributes to the G β -binding surface on G α (Fig. 1B), and changes are observed in the EPR spectra of R1 at some sites in the switch I sequence upon heterotrimer formation. In particular, residues 182R1, 184R1, and 186R1 report a decrease in side chain mobility as revealed by an increase in ΔH° and a concomitant decrease in intensity of the normalized spectra (Fig. 2A, red trace). These features are most evident for residue 184R1, which reports the addition of a new spectral component corresponding to an immobilized state of R1 (Fig. 2A, arrow). The spectral changes at 182R1 and 186R1 are more subtle, where the spectra remain dominated by components corresponding to mobile states of the nitroxide. Whereas 184R1 makes direct contact with the G β subunit, 182R1 and 186R1 project into the cleft between G α and G β without forming contacts with G β (Fig. 1C). The small decrease in mobility of these latter two residues may be accounted for by a damping of backbone motion due to G β contacts at adjacent residues (182–184). Not surprisingly, the presence of R1 at the 184 contact site reduces the affinity of *Gai* for G $\beta\gamma$. A titration of 184R1 with increasing amounts of G $\beta\gamma$ revealed that a

Fig. 2. EPR spectra for each spin-labeled *G α i* mutant along the activation pathway. The diagrams show each *G α i* conformation in a different color; the spectra are colored to match the corresponding *G α i* conformation. The spectra in each pair are normalized to the same number of nitroxide spins so that comparing relative intensities reveals changes in line width. In some cases, low and high field regions of the spectra have been expanded to clearly illustrate changes in the outer hyperfine extrema. The x axis (magnetic field) was expanded by a factor of two, whereas y-axis (intensity) expansion was arbitrary. (A) EPR spectral changes observed upon heterotrimer formation. (B) Receptor-induced changes in the heterotrimer. (C) Formation of the activated *G α i*(GTP- γ S) subunit.

10-fold excess of $G\beta\gamma$ was required to produce the full spectral change shown in Fig. 2A, whereas stoichiometric quantities sufficed for the others.

Residues 179R1 and 180R1 do not report significant changes in mobility upon addition of $G\beta\gamma$ (Fig. 2A), consistent with the crystal structure where these residues project into the intersubunit cleft far from the contact surface (Fig. 1C). The immobilized components in the spectra of 50R1, 171R1, and 187R1 show a small increase in $2A_{zz'}$ corresponding to a reduction in mobility upon heterotrimer formation (Fig. 2A). Interpreting this change is problematic for these immobilized states of R1 because the decrease in the rotational correlation time of the $G\alpha i$ subunit ($\tau_R \approx 16$ ns) upon formation of the heterotrimer ($\tau_R \approx 66$ ns) likely contributes to the observed spectral changes. On the other hand, for mobile states of R1, where the correlation time of the internal motion of R1 ($\tau_i \approx 2$ ns) is substantially shorter than the rotational diffusion time of the

G α i subunit, protein rotational diffusion does not substantially influence the spectral line shape. This point is illustrated by the absence of spectral changes upon heterotrimer formation at 179R1 and 180R1, sites where R1 has high mobility. Thus, the decreases in intensity of the mobile components in 50R1, 187R1, and 171R1 suggest decreases in mobility due to changes in protein structure. Inspection of the heterotrimer crystal structure shows that G α i binding to G $\beta\gamma$ moves the β 2-strand with residue 187R1 closer to the α 1-helix and residue 50R1 (7), which may account for the decrease in mobility of these sites in the more mobile state. No obvious structural changes near residue 171 due to heterotrimer formation are evident from a comparison of the crystal structures.

Receptor Activation-Dependent Conformational Changes. Photoactivation of rhodopsin results in G protein binding and GDP release. The R*-G protein complex containing nucleotide-free $G\alpha$ conformer, for which there is no crystal structure, is stable in the

absence of guanine nucleotides. Each of the spin-labeled mutants shows wild-type levels of binding under the conditions of these experiments (SI Fig. 4).

An interesting pattern of spectral changes involving the mobile R1 surface residues emerges upon formation of the R^{*}-G protein complex (Fig. 2B). The mobility of the nitroxide at sites 182R1 and 186R1 decreases upon receptor binding. Near the empty nucleotide-binding pocket, there are striking decreases in the mobility of R1 at residues 180 and 179. As discussed above, the decreases in the mobility of these initially mobile sites cannot be due to changes in τ_R upon formation of the R^{*}-G protein complex, but must be due to changes in the internal structure or interactions of the G α i subunit.

Changes in the complex multicomponent spectra of 50R1, 171R1, 184R1, and 187R1 also are evident upon empty complex formation. Spectra of buried residues 50R1 and 187R1 show decreases in 2Azz' that suggest increased mobility. Because this effect is opposite that expected from an increase in τ_R , these changes reflect alterations in structure consistent with decreased packing in the cavity shared by these residues. For 184R1, immobilized at the G α i-G β interface, an increase in 2Azz' is observed that may have contributions from the increase in τ_R . However, the persistence of an immobilized state of 184R1 indicates that the contact interface of the heterotrimer is retained in the empty complex. Most importantly, residue 171R1 becomes further immobilized upon binding R^{*}. Although the increases in 2Azz' of the immobilized component may contain some contribution from the increase in τ_R , the decreased intensity of the mobile component signals a decrease in mobility due to a change in the internal structure. This change in environment at 171R1 demonstrates that the receptor-mediated conformational changes are propagated to the α F-helix distant from the receptor-binding surface.

Activated G α i(GTP γ S). Addition of GTP γ S to the R^{*}-bound, nucleotide-free G protein causes a conformational change leading to the dissociation of the activated G α (GTP γ S) subunit from G $\beta\gamma$ and the receptor. Upon dissociation of G α (GTP γ S) from the complex, the EPR spectra for all sites except for 50R1 reveal an increase in R1 mobility (Fig. 2C). A comparison of the resulting G α i(GTP γ S) spectra (Fig. 2C, green traces) with those of G α i(GDP) (Fig. 2A, black traces) reveals changes that accompany activation of the G α i subunit. Because this comparison is made between species of the same molecular weight and, hence, the same τ_R , EPR spectral changes reflect exclusively changes in protein structure. For ease of comparison, overlays of the spectra for G α i(GDP) and G α i(GTP γ S) are provided in SI Fig. 5.

The spectra of 171R1, 182R1, and 184R1 are very similar or identical in the G α i(GDP) and G α i(GTP γ S) forms (Fig. 3 and SI Fig. 5). For 50R1 and 187R1, a slight mobility decrease and increase, respectively, is indicated by changes in 2Azz'. The spectra of 179R1 and 186R1 have similar line shapes in both forms but reflect an increased mobility in G α i(GTP γ S) relative to G α i(GDP). Residue 180R1 is unique among the switch I residues because R1 is substantially more immobilized in G α i(GTP γ S) compared with G α i(GDP). We consider a model to account for these differences in Discussion.

Discussion

Crystallographic studies have identified conformational changes in the switch I, II, and III sequences of G α subunits of heterotrimeric G proteins that accompany G protein activation. However, the mechanism of catalyzed nucleotide exchange is unknown, and elucidation of the mechanism requires information on the conformation of the G α subunit in the R^{*}-G protein complex. Crystal structures for the complex have not yet been reported, but data regarding the properties of the complex in solution have recently been obtained from NMR (23). The disappearance of many unassigned resonances in the 2D het-

eronuclear single quantum correlation spectra of G α in the empty complex relative to the heterotrimer was interpreted as arising from severe broadening of the resonances due to conformational exchange, presumably on the microsecond to millisecond time scale. Although the NMR data may reveal the existence of important conformational exchange processes, they have not identified the specific regions of the protein involved or the molecular details of the conformations involved. In contrast, SDSL can map conformational changes to specific regions of the protein, as illustrated in Results and discussed below.

The present SDSL study is focused on identifying structural changes in the switch I sequence in G α i accompanying complex formation and complements a similar previous study on switch II (12). For convenience of discussion, the sites examined in this study can be classified in four topographical groups based on the G protein crystal structures (Fig. 1). The first group consists of residues 182R1, 184R1, and 186R1, which are solvent-exposed in G α (GDP) and are at or near the G α -G β subunit interface in the heterotrimer. The second group is composed of residues 179R1 and 180R1 in the flexible switch I loop away from the G α -G β contact surface and close to the nucleotide-binding pocket. Residues 50R1 and 187R1 of the third group project into the interior of the protein fold of G α i(GDP) between the α 1-helix and β 2-strand. Finally, the fourth group contains only residue 171R1, located near the interdomain hinge on the α F-helix. Next, we discuss the structural features of each state of G α i with respect to these groups.

Switch I Structure and Dynamics in G α i(GDP), the Heterotrimer, and G α i(GTP γ S). The high mobility of R1 at sites 182, 184, and 186 in G α i(GDP) implies a flexible backbone structure, and, as expected, the mobility of these residues decreases upon heterotrimer formation. However, only the spectrum of 184R1 reveals a strongly immobilized component upon G $\beta\gamma$ binding that indicates direct contact with the G β subunit, whereas the remaining sites have small decreases in mobility. Modeling the R1 side chain (see Materials and Methods) at 182R1 and 186R1 in the crystal structure reveals that the nitroxides project into the cleft between G α and G β without making contact with G β (Fig. 1C), whereas 184R1 must be buried at the interface. As the subunits dissociate after GTP γ S binding, the spectra of all three residues return to a state of high mobility similar to that in the G α i(GDP) state.

In G α i(GDP), residues 179R1 and 180R1 exhibit a very high degree of side chain mobility that is relatively unaffected by heterotrimer formation, consistent with their location in the crystal structures. Interestingly, the spectra for these residues in the activated, G α i(GTP γ S) conformation are distinct from the spectra for the G α i(GDP) state and reflect structural differences due to the identity of the bound nucleotide. Indeed, 179R1 is considerably more mobile in G α i(GTP γ S) relative to G α i(GDP), a result that may be accounted for by the absence of a structural water (HOH 802; PDB ID code 1BOF) in G α i(GTP γ S), which links the backbone of 179 to the Mg²⁺ ion and the β -phosphate of GDP in G α i(GDP) (SI Fig. 6). In contrast, 180R1 is considerably more immobilized in the G α i(GTP γ S) state relative to G α i(GDP). Modeling of 180R1 in the G α i(GTP γ S) structure shows that both the disulfide and nitroxide of R1 can make direct contacts with the sulfur atom of GTP γ S, which is absent in G α i(GDP) (SI Fig. 6), thus accounting for the decreased mobility.

Residues 50R1 and 187R1 of the third group are predominately immobilized due to their location in the interior of the protein fold of G α i(GDP), but each also has a small population of a mobile state that may arise from a second rotamer of R1 (14). These mobile states decrease mobility upon heterotrimer formation. As we have discussed, the apparent decrease in mobility of the mobile component at both sites, although small, signals internal structure changes. This result may be due to the movement of the β 2-strand closer to the α 1-helix as observed in the crystal structures. The mobility difference of 50R1 and 187R1 in G α i(GTP γ S) compared with

Gai(GDP) implies structural differences involving the space between the α 1-helix and β 2-strand that cannot be specified from the limited data.

Finally, residue 171R1 in Gai(GDP) makes direct contacts with the α 1-helix across the interdomain cleft that account for the immobile component of the spectrum. Formation of the heterotrimer results in a general decrease in the mobility of this residue, identifying allosteric changes propagated from the G β interaction surface to the distant α F-helix. GTP γ S binding reverses the changes observed with heterotrimer formation, indicating that the local structure around 171R1 is similar in the two forms.

Receptor Activation-Dependent Conformational Changes Near Switch I

The GTPase and helical domains clamp down on the guanine nucleotide, and it has been speculated that to exchange guanine nucleotides there must be a conformational change that opens the cleft (3, 24, 25). Switch I is one of the linkers between the two domains and is connected to the receptor-binding domain at the C terminus and the β 2– β 3 loop by the β 2-strand. Nearly every site examined in the switch I region demonstrated receptor activation-dependent conformational changes. In addition, there are increases in the basal nucleotide exchange for sites 171R1, 182R1, and 184R1, consistent with previous studies by Majumdar *et al.* (24) showing that Gly→Pro mutations in switch I increase basal GDP release rates in a G α /Gai chimera. Comparing the heterotrimeric versus receptor-bound EPR spectra for sites 182R1, 184R1, and 186R1 near the G β interface demonstrates a clear structural rearrangement of the Gai–G β interface. Although the slight decrease in mobility of 184R1 may have contributions from slower protein rotational diffusion, the decreases in mobility of 182R1 and 186R1 unambiguously arise from changes in structure. The location of 182R1 and 186R1 in the structure (Fig. 1C) suggests that new contact interactions may be formed with G β , a contention that is supported by the reversal of the spectral changes upon the dissociation of Gai(GTP γ S). These new contact interactions would require a change in the orientation or structure of the subunits in the empty complex compared with the heterotrimer.

In addition to conformational changes at the Gai–G β interface, empty complex formation causes a marked decrease in the mobility of 179R1 and 180R1 near the nucleotide-binding pocket distant from the G β contact site. Indeed, residue 180R1 establishes new interactions that give rise to an immobilized state of the side chain. Based on the orientation of the R1 side chain, one likely interaction is with the phosphate-binding loop (P-loop) at the N terminus of the α 1-helix (Fig. 1), which would occur, for example, if switch I were to move in a direction to partially occupy the empty nucleotide-binding site. The decrease in the mobility of 179R1 may be due to damping of the backbone motion caused by contact interactions formed at 180R1 with P-loop residues.

Residues 50R1 and 187R1 show distinct increases in mobility upon formation of the empty complex that suggest a decrease in packing in the fold between the α 1-helix and β 2-strand. Each of the changes discussed above provides direct evidence for structural changes at the Gai–G β interface.

Two models have previously suggested that the activated receptor uses G $\beta\gamma$ to open the nucleotide-binding pocket for GDP release. In the lever-arm model, R* rotates G $\beta\gamma$ away from G α , pulling switches I and II along with it, thereby causing GDP release (26, 27). The gear-shift model proposes a rotation in the opposite direction that causes close-packing of switches I and II with the core of G α (28). Although the spectral changes described above do not uniquely describe a particular motion, they generally support a role for intersubunit structural rearrangements in switch I in the formation of the R*-bound, nucleotide-free G protein complex. However, the comparatively small spectral changes observed in switch II upon receptor binding seem to argue against a global rearrangement of the G α –G β interface as in the models described

Fig. 3. Opening the door for GDP release. (A) Transparent surface model of the Gi heterotrimer with the α 5/ β 6 and switch I/ α F motifs shown as ribbons. Bound GDP is shown as blue spheres buried in the binding pocket between the GTPase (gray) and helical (yellow) domains. The R*-binding surface is indicated with a dashed line. (B) Removing the side chains from the surface rendering of α 5/ β 6 and α F clearly exposes the nucleotide, suggesting that a movement that rearranges the side chains of these regions could provide a possible exit route for GDP from the interdomain cleft.

above, but rather suggest allosteric changes as a result of R* binding to G α .

Site 171R1 is perhaps the most interesting to demonstrate receptor activation-dependent conformational changes. Residue 171 is located in the α F-helix in the hinge between the helical and GTPase domains (Fig. 3). The location of GDP buried deep between these two domains apparently requires an opening in the interdomain cleft to allow GDP release, and one exciting possibility is that 171R1 senses a motion of the α F-helix directly involved in such an opening.

Although the specific structural rearrangement of switch I and contiguous α F-helix cannot be determined from the current data, the changes reported by R1 may constitute one component of a molecular mechanism leading to GDP release from the R*-G protein complex. Specifically, the data presented here suggest that structural changes at the G α –G β interface triggered by receptor binding, either through changes propagated along the α 5– β 6 loop, down the β 2-strand from the β 2– β 3 loop, or coupled through movements of G β , are propagated to the α F-helix. Previously, a rigid body movement of the α 5-helix, initiated by direct receptor interaction with the C terminus of G α , was shown to play a critical role in G protein activation (29). As demonstrated in Fig. 3, the α F-helix and the end of the α 5/ β 6-loop both serve to occlude the nucleotide-binding site. Concerted motion of the α F-helix, perhaps coupled to movement of the entire helical domain, and the α 5/ β 6 motif may thus cooperate in opening a portal for GDP release (Fig. 3). The importance of the α F region in regulating nucleotide exchange is supported by the increased basal nucleotide exchange rate produced by the T171R1 mutation (SI Fig. 4). Although 182R1 and 184R1 had similar effects on the basal exchange rates, their

distance from the nucleotide-binding pocket and solvent-facing side chain orientations suggests an indirect effect on GDP binding. Collectively, the results highlight specific allosteric changes at the distant nucleotide-binding pocket triggered by receptor binding.

Summary. To the extent that they can be compared, SDSL data on the solution structures of *Gai*(GDP), *Gai*(GDP) $\beta\gamma$, and *Gai*(GTP γ S) are in excellent agreement with details of the corresponding crystal structures. Most significantly, this study provides structural data for switch I in the empty complex formed with the activated receptor. The results identify a possible allosteric pathway propagated along switch I at the $G\alpha$ – $G\beta$ interface to the α F-helix, which, like the α S-helix and the α S/ β 6-loop, forms part of a putative entrance to the nucleotide-binding site. Together with earlier studies that identified movement of the α S-helix coupled directly to receptor binding, the data presented here provide key components of a concerted mechanism of receptor-catalyzed nucleotide exchange.

Materials and Methods

Materials. GDP and GTP γ S were from Sigma–Aldrich (St. Louis, MO). The sulfhydryl spin-label reagent, *S*-(1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl)-methanethiosulfonate, was a generous gift from Kalman Hideg (University of Pecs, Pecs, Hungary). All other reagents and chemicals were of the highest available purity.

Preparation of Rod Outer Segment (ROS) Membranes and $G\beta\gamma$ Subunits. Urea-washed ROS membranes and $G\beta_1g_1$ were prepared as previously described (28) and stored at -80°C . All ROS and $G\beta\gamma$ samples were buffer-exchanged into 20 mM Mes (pH 6.8)/100 mM NaCl/2 mM MgCl_2 /10% glycerol before EPR experiments.

Construction, Expression, and Purification of Mutant Proteins. Briefly, we used a plasmid encoding *Gai* that contained six amino acid substitutions at solvent-exposed cysteine residues (C3S–C66A–C214S–C305S–C325A–C351I) and a hexahistidine tag between amino acid residues M119 and T120 (30). This construct served as a template for introducing individual cysteine substitutions by using the QuikChange system (Stratagene, La Jolla, CA). All mutations were confirmed by DNA sequencing (DNA Sequencing Facility, Vanderbilt University). The mutant constructs were then trans-

formed in *Escherichia coli* BL21–Gold (DE3) (Stratagene), expressed, and purified as previously described (30).

Spin-Labeling, EPR Spectroscopy, and Modeling of the R1 Side Chain.

Spin-labeling was carried out in a buffer containing 20 mM Mes (pH 6.8), 100 mM NaCl, 2 mM MgCl_2 , 50 μM GDP, and 10% (vol/vol) glycerol. The *Gai* mutants were incubated with *S*-(1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl)-methanethiosulfonate at a 1:1 molar ratio at room temperature for 5 min. Under these conditions, only the most reactive cysteine residues were modified, and the remaining buried native cysteine residues were unreactive (30). Any excess spin-labeling reagent was removed by extensive washing with buffer using a 30-kDa molecular mass concentrator. For EPR spectroscopy, a series of spectra were recorded for each spin-labeled mutant. First, *Gai* mutants (30 μM) were loaded into a sealed quartz flat cell, and spectra were recorded at room temperature on an E580 spectrometer (Bruker BioSpin, Billerica, MA) using a high-sensitivity resonator at X-band microwave frequency. The data were typically averages of 20 to 50 scans. Except where noted otherwise, $G\beta\gamma$ was then added in a 1:1 molar ratio to form heterotrimers. The diluted samples were concentrated to the same concentration as the initial *Gai* mutants, and the EPR spectra were recorded both alone in solution and upon addition of urea-washed ROS membranes in the dark (150 μM). The sample was subsequently irradiated for 30 sec by using a tungsten lamp (cutoff filter; $\lambda > 500$ nm), and the EPR spectra were recorded immediately after bleaching. Finally, 200 μM GTP γ S was added to the samples, and the EPR spectra were recorded.

The R1 side chain was modeled by using rotamers defined by the dihedral angles of the first two bonds of the side chain, starting at the backbone (X_1 , X_2). The preferred rotamers, obtained in crystal structures of R1 and derivatives in T4 Lysozyme, are (-60° , -60°) and (180° , $+60^\circ$) (refs. 14 and 19 and M. Fleissner, D. Cascio, K. Hideg, and W.L.H., unpublished data). Several structures from the unpublished work that illustrate the rotamers have been deposited (PDB ID codes 1ZWN, 1ZYT, 2A4T, and 2CUU). The particular rotamer and the value of X_3 (the disulfide dihedral) were selected to minimize steric overlaps in the protein.

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