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β_2 adrenergic receptor-induced conformational changes in the heterotrimeric G protein Gs

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Summary

G protein-coupled receptors (GPCRs) represent the largest family of membrane receptors¹ that instigate signaling through nucleotide exchange on heterotrimeric G proteins. Nucleotide exchange, or more precisely GDP dissociation from the G protein α -subunit, is the key step toward G protein activation and initiation of downstream signaling cascades. Despite a wealth of biochemical and biophysical studies on inactive and active conformations of several heterotrimeric G proteins, the molecular underpinnings of G protein activation remain elusive. To characterize this mechanism we applied peptide amide hydrogen-deuterium exchange mass spectrometry (DXMS) to probe changes in the structure of the heterotrimeric G protein Gs (the stimulatory G protein for adenylyl cyclase) upon formation of a complex with agonist-bound β_2 adrenergic receptor (β_2 AR). Our studies reveal structural links between the receptor binding surface and the nucleotide-binding pocket of Gs that undergo higher levels of hydrogen-deuterium exchange (HX)

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Author Contributions

K.Y.C. prepared nucleotide-treated samples for DXMS, performed hydrogen-deuterium exchange of prepared samples, and analyzed mass spectrometry data. Performed cross-linking studies. S.G.F.R. performed the final stages of β_2 AR purification; assisted with β_2 AR and Gs protein virus production and expression in insect cell cultures; worked out conditions to form, stabilize and purify the β_2 AR-Gs complex using agonist BI-167107 and detergent MNG-3. T.L. was responsible for the overall data analysis. S.L. ran prepared samples on the mass spectrometer. B.T.D. managed Gs heterotrimer subunit virus production and titration; expressed and purified Gs protein; with K.Y.C. and T.L., he analyzed mass spectrometry data. P.S.C. provided MNG-3 detergent for stabilization of the β_2 AR-Gs complex. D.C. assisted with Gs heterotrimer expression and purification. B.K.K. was responsible for overall project strategy and management, and assisted with manuscript preparation. V.L.W.Jr. oversaw mass spectrometry experiments, and assisted with manuscript preparation. R.K.S. supervised Gs protein production, provided valuable ideas and insights into Gs structure and function, and wrote the manuscript.

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than would be predicted from the crystal structure of the β_2 AR-Gs complex. Together with x-ray crystallographic and electron microscopic data of the β_2 AR-Gs complex (ref 2 and Westfield *et al*, manuscript submitted), we provide a rationale for a mechanism of nucleotide exchange whereby the receptor perturbs the structure of the amino-terminal region of α -subunit of Gs and consequently alters the 'P-loop' that binds the β -phosphate in GDP. As with the ras-family of small molecular weight G proteins, P-loop stabilization and β -phosphate coordination are key determinants of GDP (and GTP) binding affinity.

The formation of a complex between a G protein-coupled receptor (GPCR) and a heterotrimeric G protein is responsible for the majority of transmembrane signaling in response to hormones and neurotransmitters. Heterotrimeric G proteins are composed of a nucleotide-binding α -subunit ($G\alpha$) and an obligate dimer of the β and γ -subunits ($G\beta\gamma$). In their inactive form $G\alpha$ -subunits are bound to GDP and tightly associated with $G\beta\gamma$. $G\alpha$ proteins are evolutionarily related to the ras family of G proteins but contain a small globular domain, referred to as the α -helical domain ($G\alpha$ sAH) (Fig 1a). The ras homology domain ($G\alpha$ sRas) contains most of the catalytic residues necessary for GTP hydrolysis, as well as the $G\beta\gamma$ and effector binding regions³. The structures within these regions differ between GTP- and GDP-bound states, and are appropriately termed switch regions^{4,5}.

The process of G protein activation involves formation of a complex (often referred to as a ternary complex) consisting of an agonist-occupied GPCR and a nucleotide-free heterotrimer. In a companion manuscript we present the β_2 AR-Gs crystal structure² (Fig 1b), which provides the first high-resolution snapshot of such ternary complex. However, a static view of this structure provides only limited insight into the mechanism of GDP release, the first step in G protein activation. In an effort to provide dynamic insights into the mechanism of receptor-mediated activation of Gs, the stimulatory G protein for adenylyl cyclase, we studied the effect of agonist-bound β_2 AR on the structure of Gs using peptide amide hydrogen-deuterium exchange mass spectrometry (DXMS, see Supplementary Methods). The rates of exchange of the amide hydrogens in a protein are a function of the protein's thermodynamic stability, most particularly the stability of the hydrogen bonds that each amide forms in the protein's native structure. In general, an amide hydrogen involved in an intra-molecular hydrogen bond, such as those that occur in a β -strand or α -helix in a relatively stable region of the protein, exchanges more slowly than amide hydrogens forming less stable bonds⁶⁻¹¹. Changes in exchange rates reflect the propensity to formation or disruption of these bonds, thereby providing information about protein structural changes and dynamics.

We examine the amide hydrogen exchange behavior of 1) the Gs heterotrimer alone, 2) Gs in complex with β_2 AR, 3) following uncoupling (dissociation) of the β_2 AR-Gs complex with transition state analogue, GDP/aluminum fluoride (AlF_3 or $[AlF_4]^-$), and 4) when the β_2 AR-Gs complex is exposed to GDP (Fig 2, supplemental data Fig S2-S9A). When comparing the Gs heterotrimer with the β_2 AR-Gs complex we observe marked changes in exchange rates for $G\alpha$ s, but little change in the exchange rates of $G\beta$ or $G\gamma$ (Supplemental Fig.S2-S5). In addition, we do not report exchange behavior on the β_2 AR itself since comparatively limited peptide probe sequence coverage was obtained for the receptor when we employed DXMS analysis conditions optimal for the G proteins.¹²

Transitioning from the GDP-bound heterotrimer to the nucleotide-free β_2 AR-Gs complex is associated with changes in exchange rates throughout the $G\alpha$ subunit (Fig 2a). There is a general increase in exchange in the segments that form the nucleotide binding pocket, consistent with a loss of polar interactions with GDP or as a result of alterations in protein structure following GDP dissociation. We observe reduced exchange at the C-terminal portion of the α 5-helix when bound to β_2 AR (Fig 2a and 3a,b). This is not surprising

considering the crystal structures of opsin and metarhodopsin bound to a C-terminal peptide of transducin¹³⁻¹⁵, and with Gs bound to β_2 AR (ref 2), showing that the C-terminus of G α subunits penetrates into the cytoplasmic core of the transmembrane bundle of GPCRs. This finding is also consistent with a plethora of biochemical and biophysical evidence (reviewed by Oldham *et al* 2006)¹⁶, as well as mutagenesis studies and peptide analysis that suggest that the C-terminus determines receptor coupling specificity¹⁷.

In contrast to the C-terminal region of α_5 , we observe a dramatic increase in exchange rates of peptide amides in the amino terminal segment of the α_5 -helix, suggesting an increase in the dynamics or disordering of this segment that is not evident in the structure of β_2 AR-Gs complex (Fig 2a, 3a,b and Supplemental data Fig. S11a). The β_6 - α_5 loop constitutes part of the nucleotide-binding pocket. Thus, increases in exchange at the amino terminal portion of the α_5 -helix likely reflect the sensitivity to nucleotide release (Fig 2a, 3b). The reintroduction of GDP reverses the effects observed on formation of the complex in this region (Fig 2c). These observations are consistent with EPR spectroscopy studies suggesting movement of the α_5 -helix of the G protein transducin upon interacting with photo-activated rhodopsin¹⁸.

As expected, uncoupling Gs from the β_2 AR with GDP/AlF₃ reverses all exchange changes induced by receptor binding (Fig. 2b). GDP/AlF₃ is a transition state analogue that structurally and functionally mimics GTP when bound to G proteins. Interestingly, the overall effect of adding GDP on exchange rates was similar to the effect of adding GDP/AlF₃; however, we did not observe a comparable increase in exchange at the carboxyl terminal end of the α_5 -helix (Fig 2c). These results suggest that the carboxyl terminal peptide of GDP-bound G α s may remain weakly coupled to the receptor and are consistent with crosslinking experiments performed under similar conditions showing persistent interactions between the β_2 AR and Gs in the presence of GDP (Fig. S10). It is tempting to speculate that this GDP-bound β_2 AR-Gs complex may represent a 'pre-coupled' receptor complex. Nevertheless, it is likely that the GDP-bound β_2 AR-G α s complex is not very stable since increased exchange at the α_5 -helix carboxyl terminus could be observed following longer exchange durations or incubations at higher (but still physiologic) temperature (see Supplemental Fig S2 and S10).

The most striking effect of β_2 AR-Gs complex formation on amide hydrogen exchange was observed in the β_1 -strand, which links the second intracellular loop (ICL2) of the agonist-bound receptor to the P-loop that coordinates the β -phosphate of GDP in G α s (Fig 2a, 3a,c). The large increase in exchange rate in the β_1 -strand is unexpected based on relatively small structural changes in β_1 - α_1 loop observed when comparing the structures of G α_i bound to GDP with the β_2 AR-Gs complex (Fig. 3c). The relatively minor differences observed in the crystal structures may not reflect the change required for GDP release, since the crystallization process often favors the lowest energy and most stable conformation. In contrast, DXMS experimentation interrogates the entire ensemble of native-state conformations under physiologic conditions (pH, salt, protein concentration and temperature)¹¹. Such analysis reveals that the β_1 -P-loop- α_1 region undergoes structural changes that markedly alter the stability of amide backbone hydrogen bonds.

The crystal structure of G α s-GTP γ S suggests that five amide hydrogens stabilize the backbone of the β_1 -strand of this peptidic fragment (Fig 3d and Supplemental data Fig S11b). Four of the five amides are contributed by the highly conserved R₄₂LLL₄₅ motif, found in all G protein α -subunits (See alignment in supplemental Fig S12). DXMS analysis indicates that 4-5 hydrogens rapidly exchange in this fragment. We were unable to detect fragments of the neighboring β_3 and β_4 strands by MS, but speculate that alterations in exchange might also be observed in these regions. The dramatic increase in exchange in this

highly conserved motif suggests that formation of the β_2 AR-Gs complex is associated with large changes in the structure and/or stability of the β -sheet formed by the $\beta 1$ - $\beta 5$ strands.

The increased exchange in the $\beta 1$ -strand, the P-loop and the proximal $\alpha 1$ -helix is compatible with the well-established mechanism of nucleotide exchange in the ras family of G proteins. Here the β -phosphate of GDP is wedged between the highly conserved 'P-loop' and $\alpha 1$ -helix, as well as a single Mg^{2+} ion that is coordinated by residues within the P-loop. GDP release in ras-like G proteins is manifested simply by altering Mg^{2+} coordination to the β -phosphate⁴. Although heterotrimeric G α proteins lack the dependence on Mg^{2+} for GDP binding, like all G proteins they display a 10^5 - 10^6 -fold preference for GDP over GMP⁵. The structural similarities between the ras and heterotrimeric family of G proteins suggest that the mechanism for nucleotide exchange should be similar. Thus, a critical action of the agonist-bound receptor appears to be the engagement of the N-terminus of G α , thereby altering the position and/or stability of the $\beta 1$ -strand with associated changes in the structure of the P-loop and $\alpha 1$ -helix.

DXMS analysis also reveals regions with higher exchange that extend beyond the nucleotide-binding site and encompass the entire interface between the G α sAH and G α sRas (Fig 2, 4). These data are consistent with crystallographic and electron microscopy evidence on the β_2 AR-Gs complex (ref 2 and Westfield *et al*, manuscript submitted). These studies and recent double electron-electron resonance analyses¹⁹ suggest that the G α sRas and G α sAH undergo a "clam shell-like" opening in the absence of nucleotide (see Fig 4). Nucleotide release appears to result in re-positioning of the G α sAH, but the observed slow rate of exchange within the core of the domain suggests that the domain itself is intact and folded. In the GDP- and GTP γ S-bound forms of G proteins, a highly conserved glutamate residue in the P-loop (E50 in G α s) tightly binds the highly conservative catalytic arginine residue (R201 in G α s), which is located in the G α sAH (Fig 3c and see alignment in Supplement Fig S12). However, the side chain of E50 in G α s in the receptor complex is poorly ordered in the crystal structure, displaying high B- or temperature factors. We propose that alterations in the P-loop structure from the loss of β -phosphate coordination disrupts the E50-R201 interaction and facilitates the dissociation of G α sAH from G α sRas.

In summary, DXMS analysis of the β_2 AR-Gs complex complements the crystal structure and provides essential additional mechanistic insights into the structural events linking GPCR-G protein complex formation and GDP release. The amino and carboxyl termini of G α s represent the principal structural conduits between the receptor and the nucleotide binding pocket (Fig. 5). Although it is not presently possible to determine the exact sequence of events leading to nucleotide dissociation and the formation of a stable complex, we speculate that the initial interaction may involve the carboxyl terminus of GDP-bound G α s and agonist-bound β_2 AR. Subsequent interactions between ICL2 of the β_2 AR and the α N-helix of G α s are associated with GDP release through dynamic changes in the $\beta 1$ -strand that involve disruption of a conserved hydrogen bond network and are coupled to structural changes in the highly conserved 'P-loop' that wraps around the β -phosphate of GDP. Thus, in a coordinated event, the agonist-bound receptor engages both the N-terminus and C-terminus to promote nucleotide release.

METHODS SUMMARY

DXMS studies were performed on purified Gs and β_2 AR-Gs complex in the absence or presence of guanine nucleotides. The β_2 AR-Gs complex was formed from β_2 AR and Gs protein expressed in insect cells. Samples were incubated on ice for 100, 1000 and 10000 seconds in the presence of D₂O, or H₂O then quenched, and frozen for subsequent analysis

by fragmentation and mass spectrometry. For more experimental details see Online Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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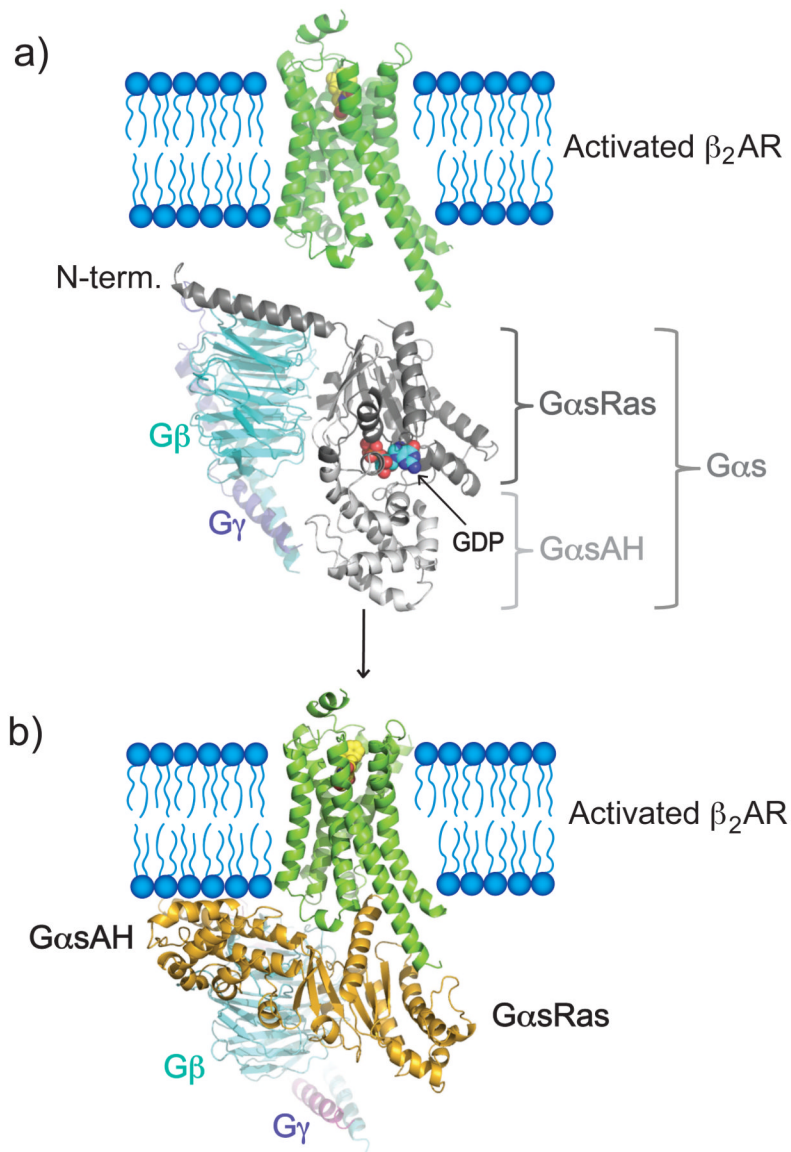


Figure 1. Receptor-mediated activation of G proteins through promoting GDP release
 Agonist (BI-167107)-bound receptor-mediated interaction with heterotrimeric G proteins promotes nucleotide (GDP) release in the G protein α -subunit. A) Structures of the $\beta_2\text{AR}$ (green) and G protein heterotrimer ($G\alpha\beta\gamma$). The nucleotide-binding $G\alpha$ -subunit (gray) is composed of a ras-homology domain ($G\alpha\text{Ras}$) and an α -helical domain ($G\alpha\text{AH}$). B) The crystal structure of BI-167107 (agonist)-bound $\beta_2\text{AR}$ with G_s reveals large domain movements in the α -subunit (yellow) of G_s that are associated with nucleotide release.

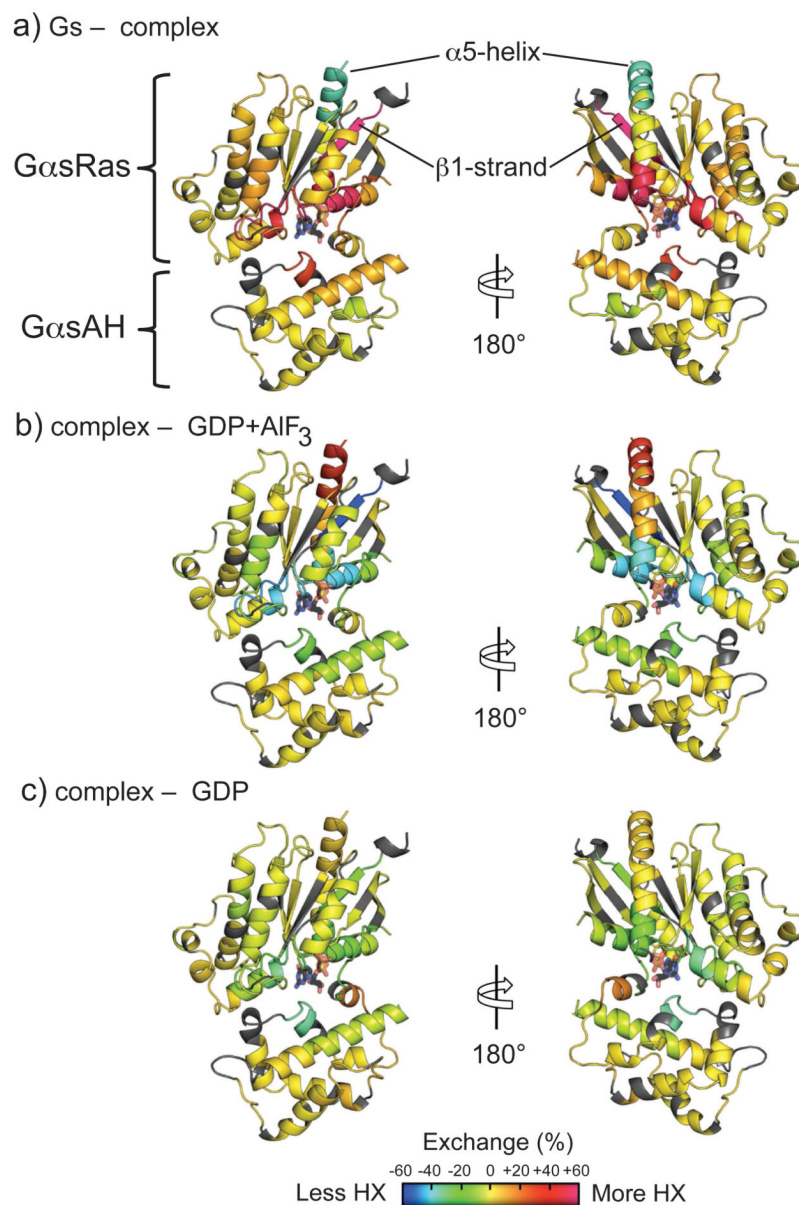


Figure 2. DXMS reveals conformational changes in the Gαs when in complex with agonist-bound β₂AR in solution

Pairwise comparisons of DXMS of Gαs under different conditions. (A) Changes in DXMS of Gαs following formation of β₂AR-Gs relative to Gαs in the Gs heterotrimer. (B) Changes in DXMS of Gαs in the β₂AR-Gs complex following dissociation of the complex with GDP/AIF₃. (C) Changes in DXMS of Gαs in the β₂AR-Gs complex following the addition of GDP alone. The changes in amide hydrogen-deuterium exchange, given as changes in the percentage of the theoretical maximum number of deuterons incorporated per peptide, were mapped on to the crystal structure of Gαs based on the GTPγS-bound form (PDB:1AZT)²⁰. Residues displaying increases (magenta) and decreases (blue) in deuterium incorporation when comparing different conditions were plotted according to the indicated heat map. Regions that are not covered are indicated in. Among 3 time points analyzed (see Figure 2S), 100 sec time points are presented.

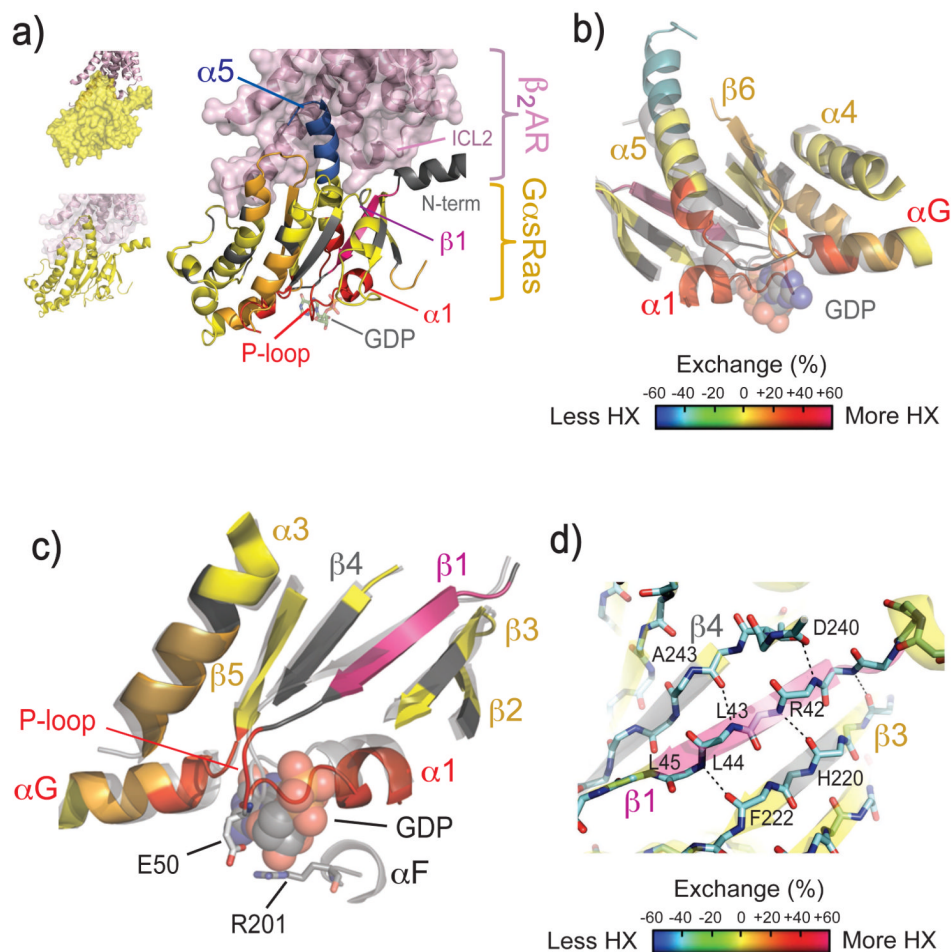


Figure 3. Agonist-bound β_2 AR-mediated activation of GDP release revealed by DXMS
 Illustrated are the conformational changes in the $G_{\alpha s}$ Ras of Gs when in complex with agonist-bound β_2 AR (mauve). Highlighted are the changes in deuterium exchange (HX), according to the indicated heat map, of key regions of $G_{\alpha s}$ Ras mapped onto the crystal structure of the complex (ref 2). (B and C) Comparison of the structure of $G_{\alpha s}$ in complex with β_2 AR with that of $G_{\alpha i}$ (the inhibitory G protein for adenylyl cyclase bound to GDP) highlighting regions of increases or decreases in HX in the β_6 -strand- α_5 helix (B) or in the β_1 -strand, P-loop and α_1 -helix (C). As above, $G_{\alpha s}$ (in B and C) is colored according to the indicated heat map representing changes in HX when comparing Gs heterotrimer with the nucleotide-free β_2 AR-Gs complex. Residues where no mass information was obtained are colored charcoal gray. The superimposed structure of $G_{\alpha i}$ bound to GDP (transparent gray) is based on the heterotrimeric GDP-bound form²¹, but illustrating the conserved glutamate (E50 in $G_{\alpha s}$) in the $G_{\alpha s}$ Ras and arginine (R102 in $G_{\alpha s}$) located in the $G_{\alpha s}$ AH. D) Five amide hydrogens, contributed by highly conserved 'RLLL' motif, are involved in stabilizing the β_1 -strand in a peptide fragment of $G_{\alpha s}$ that displays high HX.

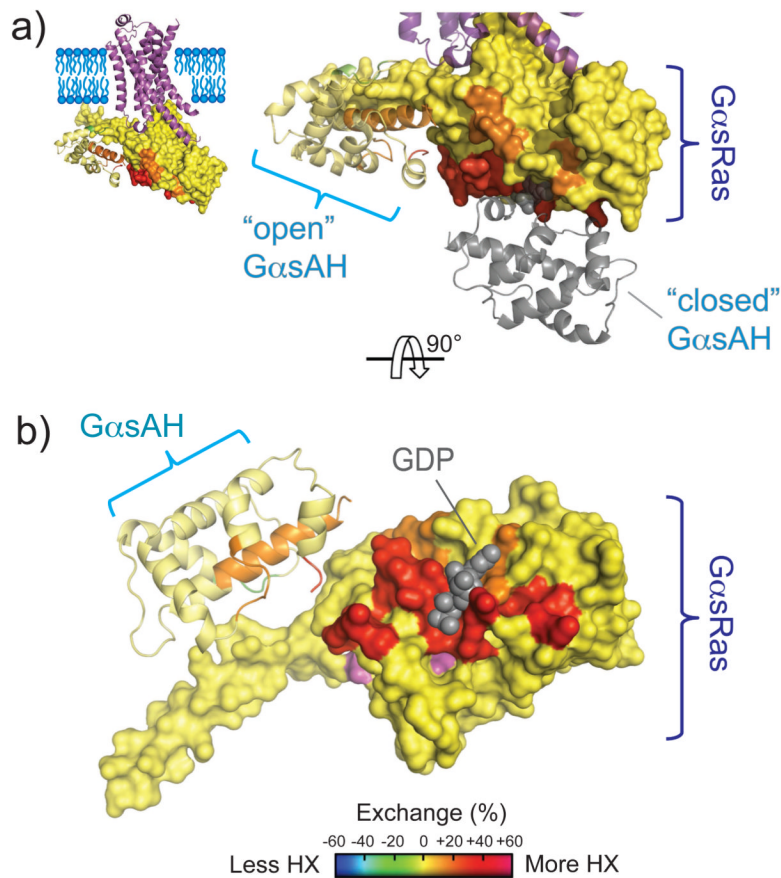


Figure 4. Deuterium exchange at the interface between the α -helical and ras homology domains
 A) Changes in HX at the interface between the G α sAH (ribbon diagram) and G α sRas (surface rendering) are mapped onto the "open" conformation of G α s observed in the β_2 AR-Gs complex (inset). In this "open" conformation, G α sAH and G α sRas are colored according to the indicated heat map. Also shown is a ribbon diagram of G α sAH in a "closed" position (gray) similar to that observed in the crystal structure of the GDP-bound G α i heterotrimer. The location of the GDP binding site is shown as spheres. B) Surface rendering of panel A) rotated back 90° to show the cytoplasmic side of the "open" conformation of nucleotide-free G α s.

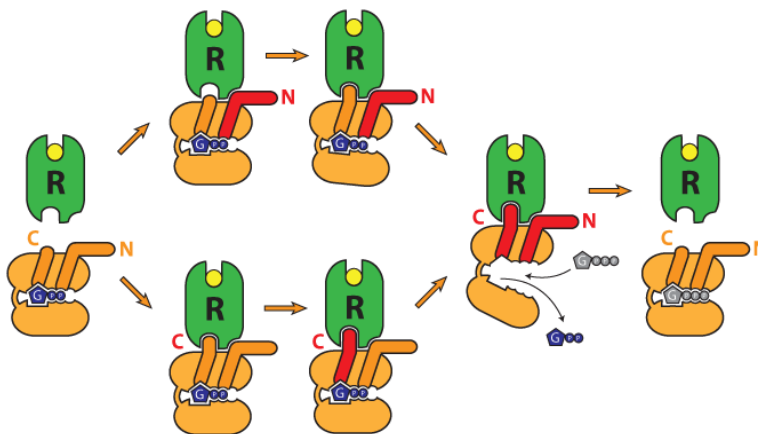


Figure 5. Mechanism for receptor-catalyzed nucleotide exchange in heterotrimeric G protein α -subunits

The step-wise dissociation of GDP from G α s (orange) by agonist-activated β_2 AR that involves the engagement of both the N- and C-terminus of G α s. The activation of Gs through an activated β_2 AR (green) results in GDP release and subsequent GTP binding. The activated receptor engages the C-terminus of the α 5-helix of G α s which undergoes a rigid-body translation upward into the receptor core and reorganizes the β 6- α 5 loop, a region that participates in purine ring binding. In a simultaneous or sequential event, ICL2 of the β_2 AR engages the N-terminus of the G α s leading to reorganization of its β 1-strand/P-loop, the loss of coordination of the β -phosphate of GDP (blue), and subsequently GDP release. The position of the N-terminal helix is aided by the G $\beta\gamma$ -subunits (not shown). The concomitant disruption of the interaction between the P-loop and G α sAH, primarily through the highly conserved R201 in the G α sAH and E50 in the P-loop, opens G α sAH allowing GDP to freely dissociate. Formation of the nucleotide-free form allows GTP (gray) to bind, resulting in reformation of the “closed” conformation, and activation of the G protein through functionally dissociating from G $\beta\gamma$ and uncoupling from β_2 AR.