Trp fluorescence reveals an activation-dependent cation-π interaction in the switch II region of $G\alpha_i$ proteins

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Abstract: Crystal structures of $G\alpha_i$ (and closely related family member $G\alpha_t$) reveal much of what we currently know about G protein structure, including changes which occur in Switch regions. $G\alpha_t$ exhibits a low rate of basal (uncatalyzed) nucleotide exchange and an ordered Switch II region in the GDP-bound state, unlike $G\alpha_i$, which exhibits higher basal exchange and a disordered Switch II region in $G\alpha_i$GDP structures. Using purified $G\alpha_i$ and $G\alpha_t$, we examined the intrinsic tryptophan fluorescence of these proteins, which reports conformational changes associated with activation and deactivation of Gα proteins. In addition to the expected enhancement in tryptophan fluorescence intensity, activation of GαGDP proteins was accompanied by a modest but notable red shift in tryptophan emission maxima. We identified a cation-π interaction between tryptophan and arginine residues in the Switch II of $G\alpha_i$ family proteins that mediates the observed red shift in emission maxima. Furthermore, amino-terminal myristoylation of $G\alpha_i$ resulted in a less polar environment for tryptophan residues in the GTPase domain, consistent with an interaction between the myristoylated amino terminus and the GTPase domain of Gα proteins. These results reveal unique insights into conformational changes which occur upon activation and deactivation of G proteins in solution.

Keywords: Gα; switch II; intrinsic fluorescence; cation-π; N-terminal myristoylation

Introduction

Activation of heterotrimeric G proteins coupled to 7-transmembrane receptors results in activated GαGTP and G$\beta\gamma$ subunits which activate downstream effectors. Hydrolysis of bound GTP to GDP facilitates re-association of GαGDP and G$\beta\gamma$, a prerequisite for productive coupling to activated receptors. The conformational changes in Gα subunits that accompany nucleotide exchange and hydrolysis are sensed by three Switch regions: Switch I, II, and III. These regions adopt distinctive conformations in crystal structures of Gα proteins bound to GDP, GDP.G$\beta\gamma$, GTPγS, and GDP-AlF_4,[1–9] facilitating interactions of Gα with G$\beta\gamma$[8,9] and a variety of effectors.[10–14] Although much of what we know about Gα structure and function has been gleaned from crystal structures, the Switch II of $G\alpha_i$ which binds G$\beta\gamma$[8,9] is disordered in $G\alpha_i$GDP crystal structures in the absence of G$\beta\gamma$, as is the N-terminus

Additional Supporting Information may be found in the online version of this article.

Abbreviations: AU, arbitrary units; BD-GTPγS, BODIPY-GTPγS; emmax, maximal emission wavelength; ex/em, excitation/emission; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor; Gi, family of G proteins coupled to inhibition of adenylyl cyclase; Gs, family of G proteins coupled to activation of adenylyl cyclase; Gt, G protein of the rod outer segment, transducin; GTP, guanosine triphosphate; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); Gα, α subunits of G proteins; G$\beta\gamma$, $\beta\gamma$ dimer of heterotrimeric G proteins which binds Gα; HI, Gαil isoform lacking several solvent-exposed cysteines; N-terminus, amino terminus; SEM, standard error of mean; wt, wild-type.

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of isolated Gα proteins. Crystallographically disordered regions in proteins are often important interfaces for protein–protein interaction. The conformation of Switch II may be important for binding to not only Gβγ, but also other Gα effectors such as GIV, a recently identified GEF for $G\alpha_i$ proteins.[15] Similarly, a peptide derived from the GoLoco region of RGS14 binds Switch II of $G\alpha_i$GDP[16] to competitively inhibit Gβγ binding. Other Gα effectors which bind Switch II of Gα proteins in a conformationally sensitive manner include adenylyl cyclase (which binds activated $G\alpha_s$)[11] and the RGS domain of RhoGEF which binds activated $G\alpha_{13}$.[17]

The Trp in Switch II (W211 in $G\alpha_i$) reports conformational changes upon activation.[20,21] The increase in emission intensity which accompanies the activation-dependent movement of the Switch II region into a hydrophobic pocket[1–7] provides a convenient and reliable method to assess the functional integrity of purified proteins.[22,23] $G\alpha_i$ contains two other Trp residues, W258 in the GTPase domain and W131 in the helical domain. Crystal structures show that the Trp in the helical domain of $G\alpha_i$ and $G\alpha_t$ proteins remains buried in both the inactive and active states,[3,4,6] and NMR studies have shown it to be refractory to activation.[24] A conformational change in a protein which relocates a Trp residue into a more solvent-excluded environment is typically accompanied by an increase in emission intensity (such as upon Gα activation) and a shift in emission maxima (em_{max}) to lower wavelengths (called a blue shift). However, in $G\alpha_i$ proteins, this activation-dependent enhancement in intensity is accompanied by a small but notable red shift in em_{max} (toward a higher wavelength). Investigation of this unexpected red shift revealed an activation-dependent cation-π interaction between Arg and Trp residues in Switch II as determined by steady-state fluorescence, and this finding was confirmed using site-directed mutagenesis. The relative differences in the Trp fluorescence of $G\alpha_i$GDP proteins before and after activation indicate an overall conformational similarity in solution between the Switch II region of $G\alpha_i$ and $G\alpha_t$ proteins.

Both Switch II and the N-terminus of Gα proteins participate in binding to Gβγ. In nature, the N-terminus of $G\alpha_i$ family proteins are permanently, cotranslationally myristoylated,[18] which enhances Gα-Gβγ association,[19] thus its conformation (along with that of Switch II) may influence the duration of Gβγ signaling. We also looked at the environment of Trp residues in myristoylated proteins, and found a myristoylation-dependent reduction in solvent exposure for Trp residues in the GTPase domain of $G\alpha_i$ proteins. Together these results shed light on conformational changes that occur in solution upon activation and deactivation of Gα subunits.

Results

There are three Trp residues in $G\alpha_i$ [Fig. 1(A)]: W211 and W258 in the GTPase domain, and a Trp in the helical domain, W131, which does not report activation-dependent changes.[4,24] Because of the well-known ability of the Trp in Switch II of Gα proteins to report such changes, functional Gα proteins typically demonstrate a minimum 40% increase in Trp emission intensity upon activation with AlF_4.[25] AlF_4 activates Gα proteins by formation of a GαGDP-AlF_4 complex which mimics the pentavalent transition state for GTP hydrolysis. In this work, we investigated the activation-dependent red shift in Trp em_{max} in $G\alpha_i$ [Fig. 1(B)], which was also present after a prolonged incubation with GTPγS (not shown), as well as upon activation of $G\alpha_i$ HI [Fig. 1(C)], a $G\alpha_i$ protein lacking several solvent exposed cysteines which does not perturb function.[26]

To gain insight into the cause of the red shift, we sought to compare structures of $G\alpha_i$ before and after activation, however, $G\alpha_i$GDP is disordered in the Switch II region.[7,27] Therefore, structures of the closely related $G\alpha_i$ family member, $G\alpha_t$ were examined, since $G\alpha_t$ shares a high sequence and structural homology with $G\alpha_i$. Comparison of the Switch II conformation in $G\alpha_t$GDP and $G\alpha_t$GDP-AlF_4 structures revealed an activation-dependent stacking of positively charged R204 (R208 in $G\alpha_i$) over the π electrons of W207 (W211 in $G\alpha_i$), reducing the distance between them by more than 2 Å [Fig. 2(A,B)]. A computational study of energetically favorable cation-π interactions determined that the CD and CZ atoms of Arg (δ-carbon and guanidinium carbon, respectively) are typically within 6 Å of the aromatic ring of Trp,[28] as is the case here [Fig. 2(A)]. In Gα proteins, this interaction is aided by a network of interactions which contribute to the positioning of the Arg-Trp pair upon activation, including a salt bridge between this Arg and a Glu adjacent to Switch III [E241 in $G\alpha_t$, Fig. 2(B), bottom panel], as well as arginine's interactions with glycine residues that contact bound nucleotide (Supporting Information Fig. 1). Structural studies show that activation tucks Switch II into a hydrophobic pocket, resulting in a closer proximity between the Arg and Trp, which may polarize Trp emission in the Switch II region. If so, this would explain the shift of the Trp em_{max} to higher, rather than lower wavelength.

Before ascertaining if the Switch II Arg was polarizing Trp emission in $G\alpha_i$ proteins, we first examined this proposal in $G\alpha_t$, which is ordered in the Switch II region in both GDP- and GDP-AlF_4-bound states. Unmyristoylated $G\alpha_t$ used in the structural determinations was prepared by treating $G\alpha_t$ obtained from rod outer segments with EndoLysC[2] to cleave the myristoylated N-terminus of $G\alpha_t$ at residue 33, followed by gel filtration. After purification, the molecular weight of the truncated Gα protein was confirmed by SDS-PAGE [Fig. 3(A), ΔNT]; this protein retained the ability to report activation-dependent changes [Fig. 3(B), inset]. We scanned the emission of the unmyristoylated $G\alpha_t$ before and after activation, and found the em_{max} underwent a red shift [Fig. 3(B)] similar to the

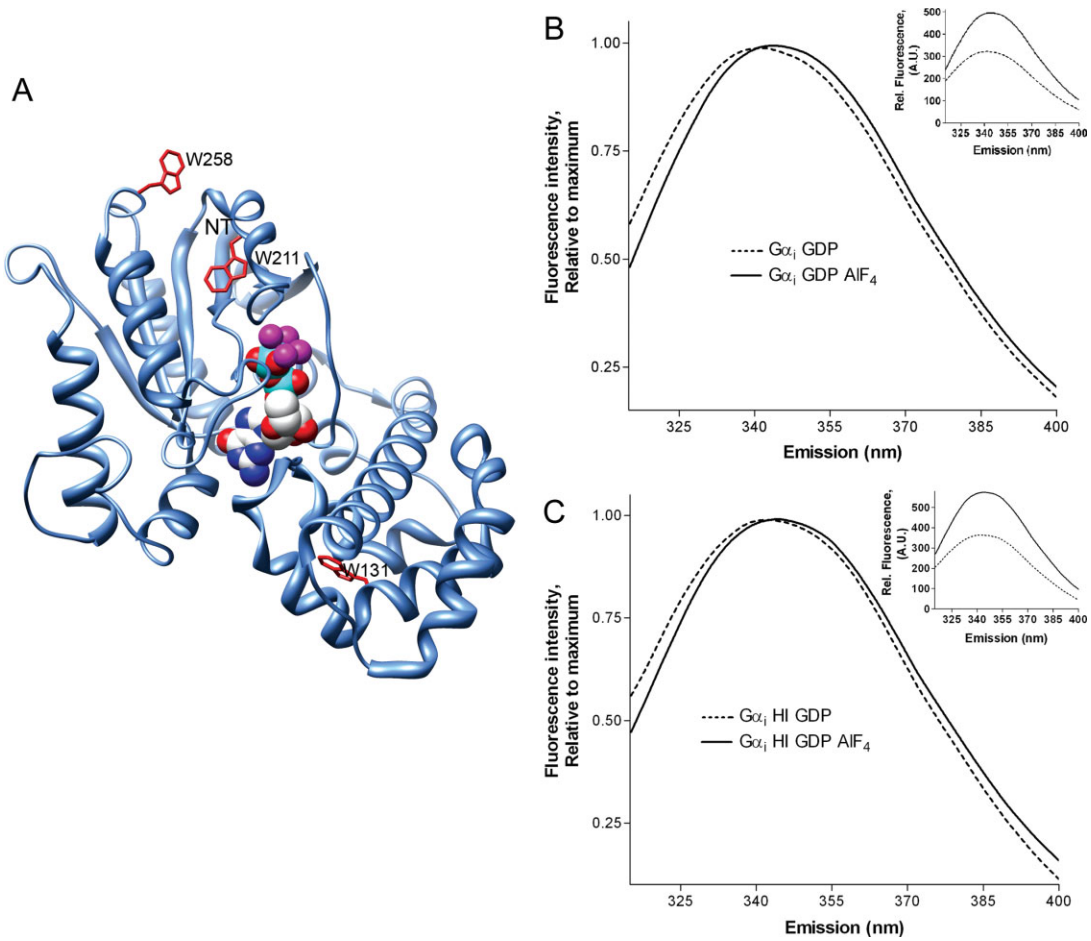


Figure 1. Intrinsic Trp fluorescence of $G\alpha_i$. (A) Location of 3 native Trp residues (red) in $G\alpha_i$GDP-AlF_4 (PDB 1GFI) with nucleotide rendered in space fill. NT, last resolved residue (33) in N-terminus. (B, C) Normalized emission of 400 n*M* $G\alpha_i$ (B, wt $G\alpha_i$; C, $G\alpha_i$ HI), ex/em 280/310–400 nm, before (dotted) and after (solid) activation with AlF_4. Control, inset: $G\alpha$ proteins demonstrate $\geq$40% increase in intrinsic Trp fluorescence upon AlF_4 activation (raw data).

one observed for $G\alpha_i$, consistent with a polarization of the Switch II Trp upon activation, linking structural changes in $G\alpha_t$ to shifts in em_{max}. In addition, comparison of unmyristoylated $G\alpha_i$ and $G\alpha_t$ proteins reveal a lower em_{max} for $G\alpha_t$ [Fig. 3(C)] regardless of activation, most likely due to the fact that $G\alpha_t$ has a Tyr instead of the more solvent exposed Trp at the position homologous to W258 in $G\alpha_i$. Trp emission dominates protein spectra between 330 and 350 nm, while Tyr has an em_{max} at 303 nm. The em_{max} of Tyr is unaffected by changes in its environment, unlike Trp which demonstrates shifts in em_{max} in response to the macroenvironment and microenvironment of tryptophan's indole ring.

Given that the activation-dependent red shift observed in $G\alpha_i$ (Fig. 1) was recapitulated in $G\alpha_t$ (Fig. 3), and in light of the associated increase in proximity between Arg and Trp in Switch II of $G\alpha_t$ (Fig. 2), the red shift we observed in $G\alpha_i$ may indicate a similar geometry. If so, R208 and W211 of $G\alpha_i$ would be predicted to play critical roles. To investigate this, we examined the em_{max} of 4 $G\alpha_i$ HI proteins: W211C, R208C, R208L, and W258F, residues all located in the GTPase domain, where activation-dependent changes are known to occur. $G\alpha_i$ HI proteins were used in this study because they have been shown to tolerate cysteine mutations in the Switch II region[29] and elsewhere throughout the protein.[26,29,30] Residues W211 and R208 are part of the Switch II region of $G\alpha_i$, and W258 is located on a more solvent exposed loop adjacent to Switch II in the GTPase domain [Fig. 1(A)].

Although the $G\alpha$ proteins used in this study generally demonstrated a minimum of 40% increase in Trp intensity upon activation, mutation of residues implicated in the cation-π interaction precluded assessment of functionality by this method. Therefore, we used an extrinsic probe linked to GTPγS to confirm the ability of these proteins to exchange nucleotide. Uncatalyzed exchange of GDP for BD-GTPγS is reported by an increase in emission from BD-GTPγS that occurs upon its binding to $G\alpha_i$ subunits.[31] Because $G\alpha_i$ subunits are known to bind to BD-GTPγS at a slower rate and with a lower affinity than unlabeled GTPγS,[31] overall BD-GTPγS binding was measured after full exchange was allowed to occur. The indicated $G\alpha_i$ proteins showed the ability to exchange GDP for

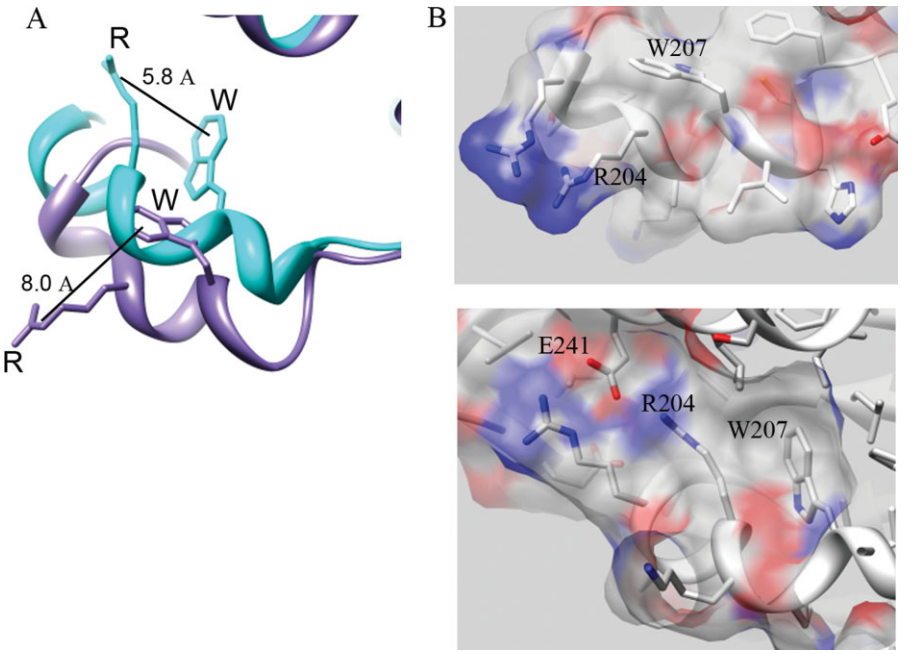


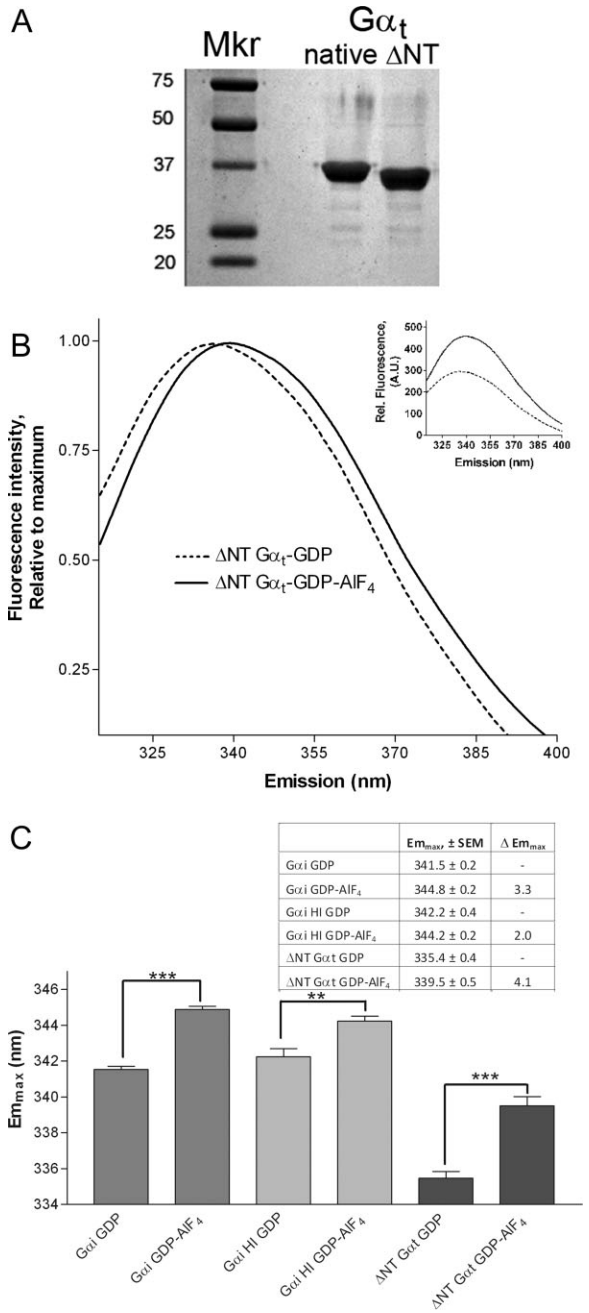
Figure 2. Activation-dependent increase in proximity between Arg and neighboring Trp in Switch II of $G\alpha_t$. (A): Switch II Trp in $G\alpha_i$ family member $G\alpha_t$ is 2.2 Å closer to positively charged Arg upon activation, $G\alpha_t GDP\text{-}AlF_4$ (PDB file 1TAD) (teal) overlay with $G\alpha_t GDP$ (PDB file 1TAG) (purple). Note residues R204 and W207 in $G\alpha_t$ correspond to residues R208 and W211 in $G\alpha_i$. (B, top panel): Switch II of $G\alpha_t GDP$ shown with surface rendered. (B, bottom panel): Switch II tucks into protein upon activation, shown with surface of $G\alpha_t GDP\text{-}AlF_4$ rendered.

BD-GTPγS [Fig. 4(A), solid bars], which was eliminated by pre-boiling these proteins [Fig. 4(A), far right bars]. The mutant proteins were also monodisperse on gel filtration, exhibited retention time on gel filtration chromatography according to their predicted molecular weight, and migrated essentially the same as wild-type proteins on SDS PAGE, consistent with proper expression and folding of these proteins.

Since AlF_4 binding to wild-type $G\alpha_i GDP$ is known to reduce nucleotide exchange with GTP analogs due to additional stabilizing contacts introduced by binding AlF_4,[4,32] we next measured nucleotide exchange using Gα proteins pre-activated by AlF_4 [Fig. 4(A), striped bars]. The stabilizing contacts introduced by AlF_4 reduced binding of BD-GTPγS by $G\alpha_i$ HI as expected, and did so to a somewhat lesser extent in the R208 mutants, however mutation of W211 resulted in the complete loss of AlF_4-mediated stabilization of bound GDP. This protein exchanged GDP for BD-GTPγS regardless of activation by AlF_4 [Fig. 4(A), striped bars].

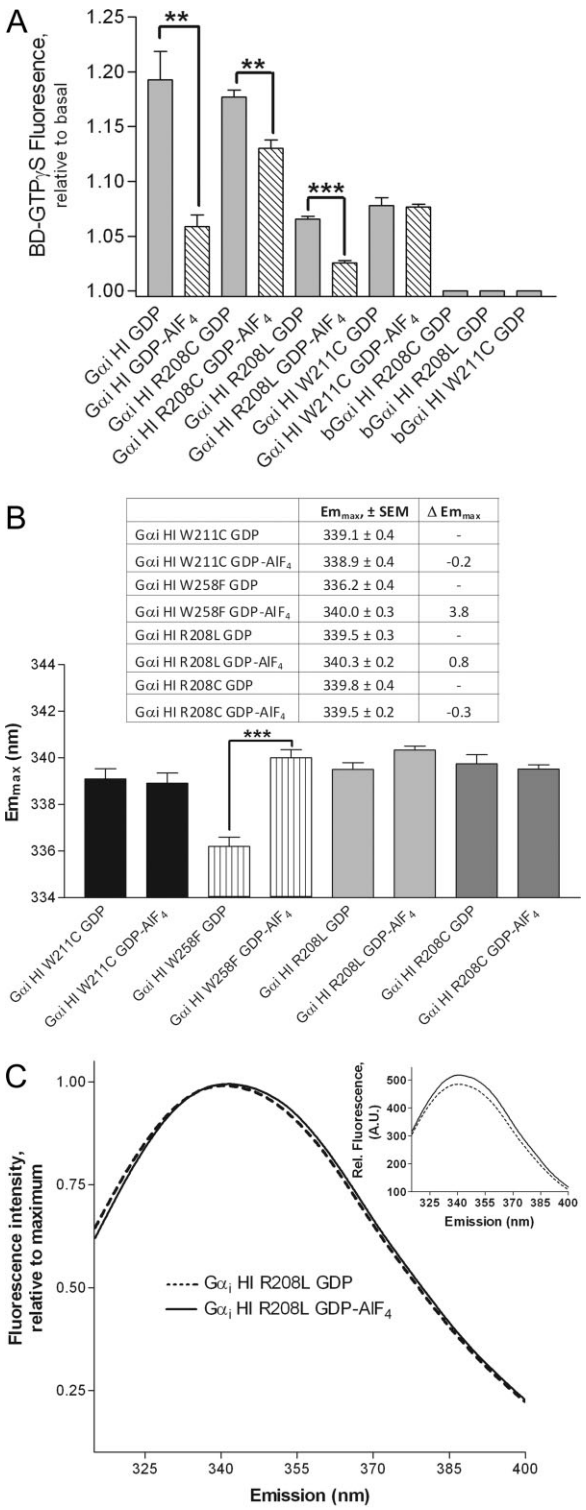
Mutation of either R208 or W211 attenuated the red shift upon activation [Fig. 4(B,C)], despite the ability of these proteins to exchange nucleotide [Fig. 4(A)]. Mutation of the same Switch II Trp in $G\alpha_o$ also prevents detection of activation by Trp fluorescence, without preventing activation-dependent conformational changes or the binding to labeled GTPγS.[33] Both R208 and W211 are direct participants in the cation-π interaction and play a role in stabilization of the Switch II region upon activation through a combination of electrostatic, hydrophobic and van der Waals interactions. Mutation of W258 did not impair the activation-dependent red shift in em_{max} [Fig. 4(B)], and it resulted in a lower em_{max} than the parent $G\alpha_i GDP$ HI protein [Fig. 4(B) vs. Fig. 3(C)], indicating a high degree of solvent exposure for this Trp. These studies were performed in the absence of Gβγ and receptor which contain numerous Trp residues that obscure detection of changes in Gα emission by increasing background signal.

Using $G\alpha_i$ proteins coexpressed with N-myristoyl transferase, we found that N-terminal myristoylation reduced the activation-dependent red shift in wild-type $G\alpha_i$, and eliminated it in native $G\alpha_t$ [Fig. 5(A) vs. Fig. 3(C)], without impairing activation-dependent increases in intensity [Fig. 5(B), shown for $G\alpha_t$]. Interestingly, myristoylation also reduced the em_{max} of GDP-bound $G\alpha_i$ proteins with a native Trp at position 258 [Fig. 5(C)], but did not have the same effect on Gα proteins lacking this or an equivalent Trp, $G\alpha_i$ HI W258F, and $G\alpha_t$. Thus, W258 in the GTPase domain senses myristoylation in GDP-bound $G\alpha_i$ subunits. Myristoylation also decreases the em_{max} of activated, wild-type $G\alpha_i$ and $G\alpha_t$ [Fig. 5(D)], consistent with a myristoylation-dependent modulation of the cation-π interaction in these proteins, either directly or indirectly, however, the exact nature of the interaction has yet to be determined. Taken together, myristoylation has effects on both W258 and W211, which are located near the last resolved residues in the N-terminus of $G\alpha_i$ [Fig. 1(A)]. These data are in agreement with a



	Em_{max}, ± SEM	Δ Em_{max}
Gαi GDP	341.5 ± 0.2	-
Gαi GDP-AlF_4	344.8 ± 0.2	3.3
Gαi HI GDP	342.2 ± 0.4	-
Gαi HI GDP-AlF_4	344.2 ± 0.2	2.0
ΔNT Gαt GDP	335.4 ± 0.4	-
ΔNT Gαt GDP-AlF_4	339.5 ± 0.5	4.1

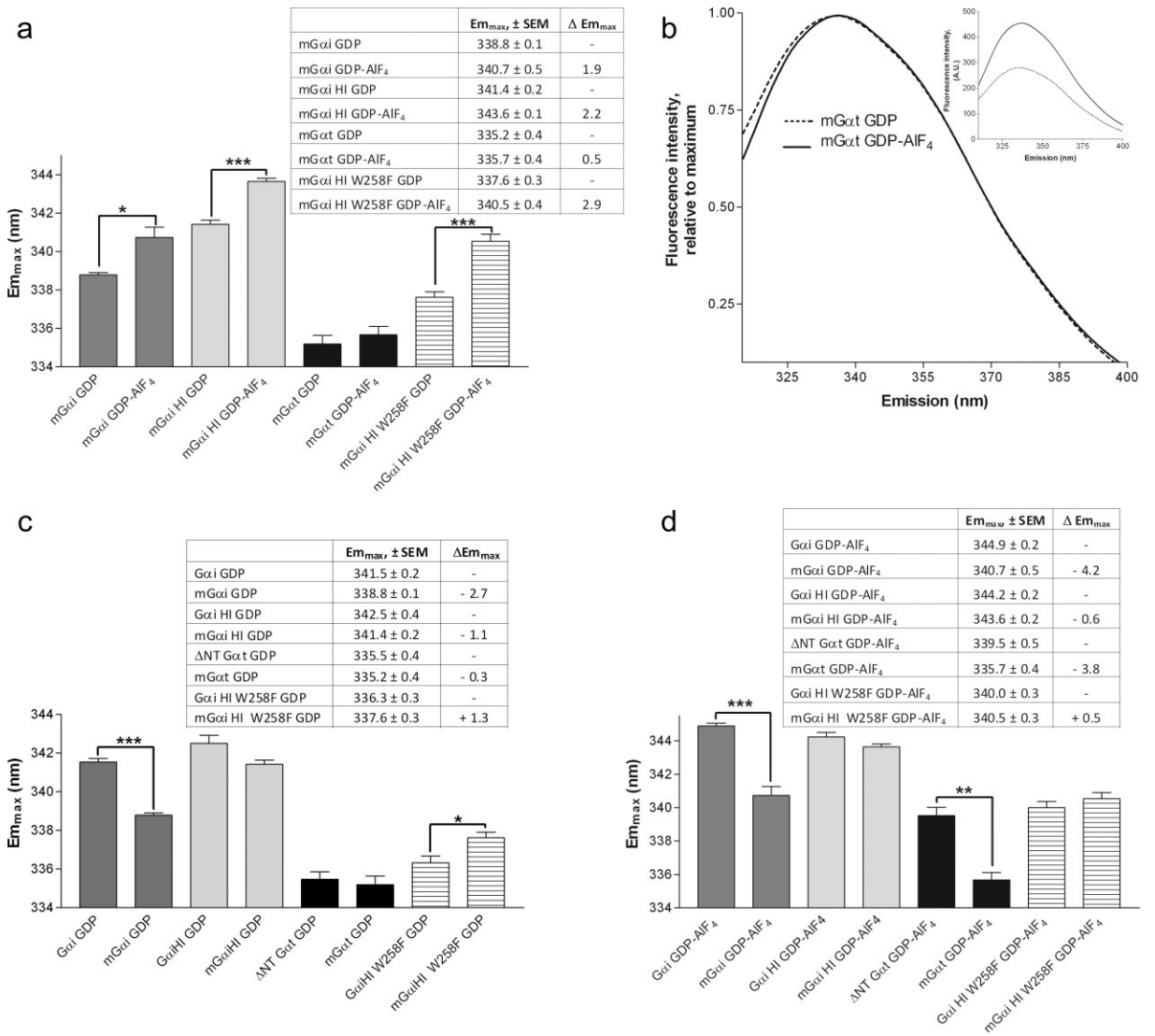
Figure 3. Trp fluorescence of unmyristoylated proteins. (A) Myristoylated $G\alpha_t$ (native) is enzymatically cleaved at the N-terminus to yield the unmyristoylated ΔNT $G\alpha_t$ protein used in structure determination.[6] (B) Emission of 500 n*M* ΔNT-$G\alpha_t$ protein was scanned as above before (dotted line) and after (solid) activation with AlF_4 as in Figure 1. Control, inset: ΔNT-$G\alpha_t$ demonstrated ≥40% increase in Trp fluorescence upon AlF_4 activation. (C) Summary of em_{max} before and after activation for indicated Gα proteins ($n \geq 3$, ±SEM; statistically significant changes noted by asterisk(s), $^{***}P < 0.001$, $^{**}P < 0.01$). Dark gray bars, far left, $G\alpha_i$; light gray, center, $G\alpha_i$ HI; black, far right, Δ$NTG\alpha_t$.



	Em_{max}, ± SEM	Δ Em_{max}
Gαi HI W211C GDP	339.1 ± 0.4	-
Gαi HI W211C GDP-AlF_4	338.9 ± 0.4	-0.2
Gαi HI W258F GDP	336.2 ± 0.4	-
Gαi HI W258F GDP-AlF_4	340.0 ± 0.3	3.8
Gαi HI R208L GDP	339.5 ± 0.3	-
Gαi HI R208L GDP-AlF_4	340.3 ± 0.2	0.8
Gαi HI R208C GDP	339.8 ± 0.4	-
Gαi HI R208C GDP-AlF_4	339.5 ± 0.2	-0.3

Figure 4. A: Extrinsic nucleotide exchange. Solid bars (controls): Gα proteins exchange GDP for BD-GTPγS, in contrast to heat-denatured Gα (boiled, last three bars). Striped bars: nucleotide exchange in Gα proteins that were preactivated by AlF_4 before assay. B,C: Red shift is markedly reduced by mutation of either W211 or R208, unlike mutation of Trp outside of Switch II, W258 (A, B: $n \geq 3$, ±SEM; $^{**}P < 0.01$; $^{***}P < 0.001$).

previous model suggesting the myristoylated N-terminus of $G\alpha_i$ proteins interact with residues on the surface of $G\alpha_i$,[18,27] with myristoylation-dependent effects detected for Trp residues W211 and W258, located in



	Em_{max}, ± SEM	Δ Em_{max}
mGαi GDP	338.8 ± 0.1	-
mGαi GDP-AlF_4	340.7 ± 0.5	1.9
mGαi HI GDP	341.4 ± 0.2	-
mGαi HI GDP-AlF_4	343.6 ± 0.1	2.2
mGαt GDP	335.2 ± 0.4	-
mGαt GDP-AlF_4	335.7 ± 0.4	0.5
mGαi HI W258F GDP	337.6 ± 0.3	-
mGαi HI W258F GDP-AlF_4	340.5 ± 0.4	2.9

	Em_{max}, ± SEM	ΔEm_{max}
Gαi GDP	341.5 ± 0.2	-
mGαi GDP	338.8 ± 0.1	- 2.7
Gαi HI GDP	342.5 ± 0.4	-
mGαi HI GDP	341.4 ± 0.2	- 1.1
ΔNT Gαt GDP	335.5 ± 0.4	-
mGαt GDP	335.2 ± 0.4	- 0.3
Gαi HI W258F GDP	336.3 ± 0.3	-
mGαi HI W258F GDP	337.6 ± 0.3	+ 1.3

	Em_{max} ± SEM	Δ Em_{max}
Gαi GDP-AlF_4	344.9 ± 0.2	-
mGαi GDP-AlF_4	340.7 ± 0.5	- 4.2
Gαi HI GDP-AlF_4	344.2 ± 0.2	-
mGαi HI GDP-AlF_4	343.6 ± 0.2	- 0.6
ΔNT Gαt GDP-AlF_4	339.5 ± 0.5	-
mGαt GDP-AlF_4	335.7 ± 0.4	- 3.8
Gαi HI W258F GDP-AlF_4	340.0 ± 0.3	-
mGαi HI W258F GDP-AlF_4	340.5 ± 0.3	+ 0.5

Figure 5. Effect of myristoylation on em_{max}. (A) Comparison of em_{max} of myristoylated proteins before and after activation. (B) Normalized spectra of $G\alpha_t$ (native, myristoylated) before and after activation with AlF_4. Inset: raw data before and after activation. (C,D) Effect of myristoylation on em_{max} of GDP (C) and GDP-AlF_4 bound proteins (D). (A, C, D: $n \geq 3$, ± SEM; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

and near the Switch II region in the GTPase domain of $G\alpha_i$ proteins.

These results suggest a model whereby upon GTP hydrolysis, $G\alpha_i$GDP adopts a conformation in the Switch II region [Fig. 6(A,B)] which resembles the ordered Switch II region seen in structures of $G\alpha_t$GDP [Fig. 6(B), purple]. The red shift upon activation provides information about the position of $G\alpha_i$ Switch II residues in the GDP-bound state relative to that in the GTP-bound state, indicating a qualitatively similar distancing of the Trp and Arg in the Switch II of GDP-bound $G\alpha_i$ and $G\alpha_t$ proteins after GTP hydrolysis has occurred. The activation-dependent cation-π interaction between R208 and W211 in $G\alpha_i$ (separated by one turn of the Switch II α-helix) is stabilized by a network of interactions, including a conserved pair of flexible glycines, G202 and G203 (Supporting Information Fig. 1), which allosterically link the Switch II region to activation-dependent changes in the nucleotide binding site. Computational analysis using the program CaPTURE[28] estimates the strength of this interaction to be approximately −2.0 kcal/mol, which reflects both electrostatic and van der Waals contributions, resulting in an energetically relevant interaction between these residues.

Discussion

This study provides insights into the Switch II conformation of $G\alpha_i$GDP in solution. Crystal structures of Gα proteins demonstrate that upon activation, the Switch II tryptophan moves into a more solvent-protected environment, resulting in enhanced Trp emission intensity, and a small but unexpected red shift in em_{max}. This was originally noted without elaboration

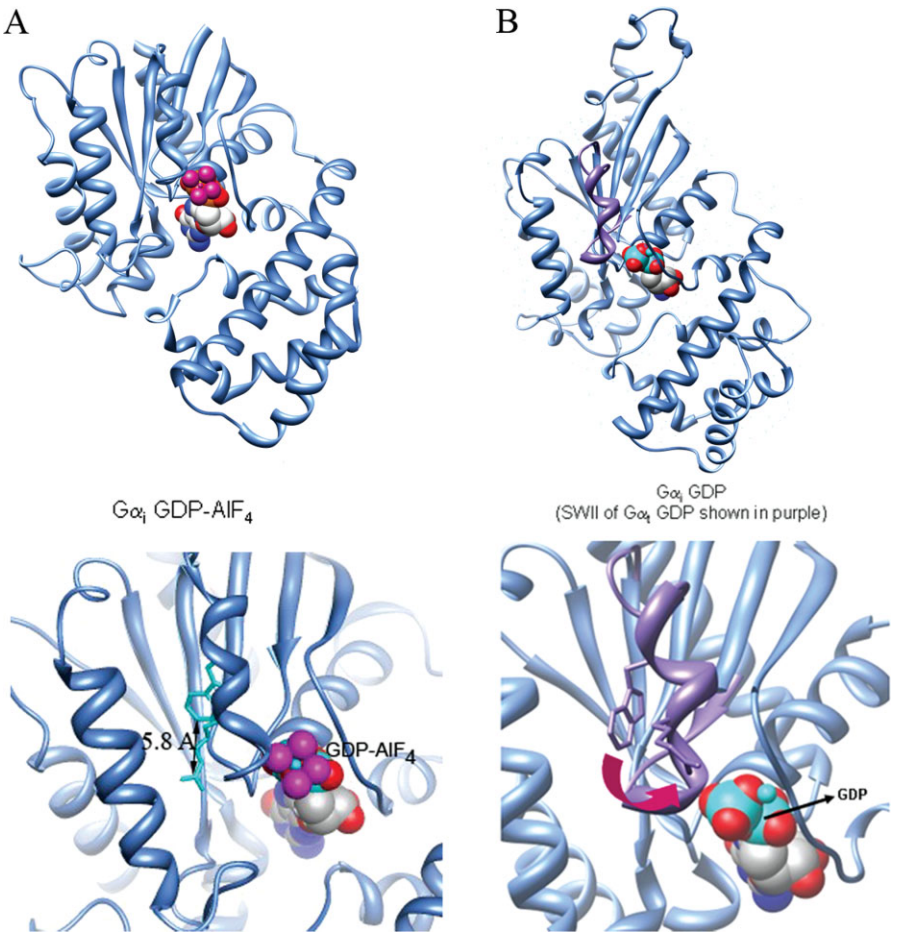


Figure 6. Model. (A) Crystal structures of activated $G\alpha_i$ (PDB file 1GFI, top panel, overview; bottom panel, Switch II region enlarged, nucleotide shown in spacefill) demonstrate that the Trp π electrons are within 6 Å from the positively charged Arg in the Switch II helix. (B) Ordered regions of $G\alpha_i$GDP (PDB file 1BOF) shown in blue, Switch II region of $G\alpha_t$GDP in purple (from structural alignment of PDB file 1TAG and 1BOF), and nucleotide shown in spacefill. Top, overview; bottom panel, enlarged, depicting spatial separation of 8 Å or more between the Switch II Arg and Trp after GTP hydrolysis has occurred.

in one of the first reports of Gα protein isolation.[23] We used a combination of mutational and biochemical approaches to identify the cause of this shift, an activation-dependent cation-π interaction, mediated by Arg and Trp in the Switch II helix of $G\alpha_i$ and $G\alpha_t$ proteins. The homology between $G\alpha_i$ and $G\alpha_t$ was used as a link between this shift and Switch II conformation, as the Switch II of $G\alpha_t$GDP is ordered in crystal structures, unlike $G\alpha_i$GDP. These solution studies indicate a relatively similar orientation of Arg and Trp in Switch II of $G\alpha_i$ and $G\alpha_t$ proteins both before and after activation.

A recent study indicated that almost 40% of energetically relevant cation-π interactions involve an Arg-Trp pair,[28] and many of these help stabilize α-helices, as in the case of Gα proteins. The majority position Trp's aromatic ring 6 Å or less from the CD and CZ atoms of Arg, over which the charge is distributed.[28] In $G\alpha_i$, the CZ of R208 moves to within 6 Å of the aromatic ring of W211 upon activation, with the CD of R208 within 5 Å of the ring, polarizing Trp emission. In $G\alpha_t$, this results in a 2.2 Å reduction in the distance between the Arg-Trp pair. Similarly, in the protein ricin, ligand binding results in a 1.7 Å reduction between its Arg-Trp pair and a 3–4 nm red shift.[34] Common cation-π interactions[35,36] position the cation parallel to the plane of tryptophan's ring of p-orbitals.[37] Energetically relevant Arg-Trp interactions are estimated at -2.9 ± 1.4 kcal/mol,[28] which includes the −2.0 kcal/mol calculated for the Arg-Trp pair in $G\alpha_i$, in agreement with a proposed cation-π interaction suggested from comparison of $G\alpha\beta\gamma_i$ and $G\alpha_i$GTPγS structures.[36] Van der Waals and hydrophobic contacts along the cation side chain contribute to the favorable energetics of the Arg-Trp pair, along with charge delocalization which maximizes Arg's interaction with Trp's indole ring. In addition, Arg often forms conventional hydrogen bonds with other residues while simultaneously interacting with Trp,[38–40] as seen in the activation-dependent interaction of R208 with E245 just next to Switch III, which allosterically links changes in nucleotide binding to Switch III conformation in $G\alpha_i$ proteins.

Residues R208 and W211, as well as G202 and G203, are highly conserved among G proteins. Mutation

of W211 abrogates the stabilizing influence normally imparted by formation of a GDP-AlF_4 complex. The structure of $G\alpha_i$GDP-AlF_4 shows that W211 is hydrogen bonded to G202, with G203 making hydrogen bonds to AlF_4 and R208, thus allosterically linking Switch II conformation to nucleotide binding. The flexibility of these conserved glycines may also facilitate Q204's interactions with the catalytic water molecule during GTP hydrolysis, which together with R178, Q171 and T181 help orient this water toward the developing positive charge on the γ-phosphate.[4] In our study, GTPγS binding also resulted in red-shifted Trp emission, as interactions that the backbone amide hydrogens of G202 and G203 also make with the γ phosphate help bring R208 and W211 within range to polarize W211 emission. Altogether, a concerted network of allosteric interactions between these conserved residues and others in the GTPase domain may drive Switch II conformation during GTP binding and hydrolysis.

Despite structural similarities between $G\alpha_i$ and $G\alpha_t$, the magnitude of the red shift differs somewhat between these proteins. In unmyristoylated Gα proteins, the relatively larger red shift for $G\alpha_t$ is likely due to an additional more solvent exposed Trp present in $G\alpha_i$ proteins, W258, that is absent in $G\alpha_t$. Mutation of W258 in $G\alpha_i$ HI reduces its em_{max} and results in a $G\alpha_t$-like emission profile, due to the location of W258 on a solvent exposed loop of $G\alpha_i$, and indicating a qualitatively similar environment for Switch II tryptophans in $G\alpha_t$ and $G\alpha_i$ proteins in solution. The conformation of Switch II after GTP hydrolysis (before Gβγ binding) may be important in regulation of basal signaling, evidenced by a peptide that binds Switch II and peels it away from the core of $G\alpha_i$-GDP, increasing nucleotide exchange.[41] We recently determined the structure of a $G\alpha_i$ protein with mutations near the receptor-binding carboxy terminus which displayed elevated nucleotide exchange and altered Switch II conformation,[32] consistent with an allosteric linkage between receptor binding, Switch II conformation and nucleotide exchange.

Not reflected in any Gα structure is N-terminal myristoylation, which has been shown in some systems to act as a myristoyl switch,[42,43] with effects on spatial and temporal regulation of signaling[44] in the visual system. Our previous work suggested an immobile and solvent excluded environment for myristoylated N-terminal residues even in the absence of Gβγ, using a series of extrinsic probes linked to specific N-terminal residues in $G\alpha_i$ HI proteins.[18] The current study has uncovered an effect of N-terminal myristoylation on Trp residues far removed in sequence from the N-terminus, suggesting an interaction of the myristoylated N-terminus with or near Trp residues in the GTPase domain. Mutation of W258 to Phe eliminates its contribution to overall Trp emission, but the preservation of a hydrophobic residue at this position likely does not impair any potential interactions that residue 258 may make with the myristoylated N-terminus. Although myristoylated proteins exhibited the requisite activation-dependent increases in Trp intensity upon activation, indicative of the movement of the Switch II Trp into a hydrophobic pocket, the associated red shifts were reduced by myristoylation of $G\alpha_i$ and eliminated in myristoylated $G\alpha_t$, indicating a myristoylation-dependent modulation of the cation-π interaction. Together these results shed light on the environment of the Switch II region of $G\alpha_i$ proteins before and after activation, and suggest a conformationally sensitive effect of N-terminal myristoylation on residues in the GTPase domain of Gα proteins, consistent with an intramolecular binding site for the myristoylated N-terminus of $G\alpha_i$ family proteins.

Materials and Methods

Materials

GDP and GTPγS were purchased from Sigma-Aldrich (Milwaukee, WI). BODIPY FL-GTPγS, thioester, was purchased from Invitrogen (Madison, WI). The endoproteinase EndoLysC was purchased from Calbiochem (San Diego, CA). All other reagents and chemicals were of the highest available purity.

Preparation of Gα proteins

$G\alpha_t$ was obtained and prepared as described previously.[2] $G\alpha_{il}$ and $G\alpha_{il}$ HI were used as is or as a basis for site-specific mutagenesis in selected residues. Construction, expression, and purification of $G\alpha_{il}$ and $G\alpha_{il}$ HI proteins (unmyristoylated and myristoylated) were performed as described previously.[18,26] Briefly, a parent $G\alpha_{il}$ HI lacking solvent-exposed cysteines which was previously shown to have properties similar to wild type[26] was generated with an expression vector encoding rat $G\alpha_{i1,}$ with the following amino acids substituted: (C66A-C214S-C305S-C325A-C351I), a hexahistidine tag inserted between amino acid residues M119 and T120, and the Switch II mutants described previously have an alanine at residue 3, which does not perturb function.[26,29] Mutations were introduced using the QuickChange system (Stratagene, La Jolla, CA); DNA sequencing confirmed all mutations. Proteins were expressed with N-myristoyl transferase to obtain myristoylated protein, and the same protein expressed in the absence of the N-myristoyl transferase vector to produce unmyristoylated proteins. Proteins were expressed and purified as detailed previously[18] in *E. coli* BL21-Gold (DE3) with or without a N-myristoyl transferase (NMT) vector pbb131, which also encodes kanamycin resistance for selection purposes (NMT vector generously provided by M. Linder, Washington University). Coomassie staining of urea SDS PAGE gels[45] demonstrate $G\alpha_i$ proteins are fully myristoylated[18] when coexpressed with the NMT vector. Purified proteins were stored at −80°C in buffer A

containing 50 m*M* Tris, 100 m*M* NaCl, 2 m*M* $MgCl_2$, 10 μ*M* GDP, pH 7.5, and 10% (v/v) glycerol; wild-type Gα containing native, solvent-exposed cysteines was additionally supplemented with 5 m*M* β-mercaptoethanol or 1 m*M* DTT before storage at −80°C. After purification, all proteins used in this study were greater than 85% pure, as estimated by Coomassie staining of SDS-PAGE gels.

Intrinsic Trp fluorescence

Proteins without mutations in Switch II demonstrated ≥40% increase in intrinsic Trp emission upon AlF_4 activation, relative to emission in the GDP-bound state, as previously described.[20] Briefly, Gα proteins (200 n*M*) in 50 m*M* Tris, 100 m*M* NaCl, 1 m*M* $MgCl_2$, pH 7.5 containing 10 μ*M* GDP were monitored by excitation/emission 280/340 nm before and after the addition of AlF_4 (10 m*M* NaF and 50 μ*M* $AlCl_3$) using a Varian Cary Eclipse Fluorometer. For proteins with mutations in Switch II region which impair the ability to report activation-dependent changes via intrinsic fluorescence, an extrinsic fluorescence using BD-GTPγS was used to assess ability to undergo activation-dependent changes (see below).

Nucleotide exchange of GDP and GDP-AlF_4 as measured by BODIPY-GTPγS fluorescence

Basal nucleotide exchange was measured as fold-increase in emission of 1 μ*M* BODIPY-GTPγS (BD-GTPγS, λ_{ex} = 490 nm, λ_{em} = 515 nm) in buffer containing 50 m*M* Tris, 100 m*M* NaCl, 1 m*M* $MgCl_2$, 10 μ*M* GDP, pH 7.5, at 21°C, in the presence and absence of 75 μ*M* AlF_4 before and 60 min after the addition of $G\alpha_i$ subunits (200 n*M*) to the cuvette containing BD-GTPγS. Boiled proteins (b) were heated at 95°C for 5 min before analysis.

Structural analysis/depictions

Computational determination of the energetics of the cation-π interaction in 1GFI was determined using the CaPTURE program.[28] Molecular graphics images were produced using the UCSF Chimera package.[46,47] Graphs and statistical analysis were performed using GraphPad Prism 4.0, GraphPad Software, San Diego, California.

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