Uncoupling conformational change from GTP hydrolysis in a heterotrimeric G protein α-subunit

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Heterotrimeric G proteins α (Gα) subunits possess intrinsic GTPase activity that leads to functional deactivation with a rate constant of ∼2 min⁻¹ at 30°C. GTP hydrolysis causes conformational changes in three regions of Gα, including Switch I and Switch II. Mutation of G202A in Switch II of Gα1 accelerates the rates of both GTP hydrolysis and conformational change, which is measured by the loss of fluorescence from Trp-211 in Switch II. Mutation of K180P in Switch I increases the rate of conformational change but decreases the GTPase rate, which causes transient but substantial accumulation of a low-fluorescence Gα1-GTP species. Isothermal titration calorimetric analysis of the binding of (G202A)Gα1 and (K180P)Gα1 to the GTP-activating protein RGS4 indicates that the G202A mutation stabilizes the pretransition state-like conformation of Gα1 that is mimicked by the complex of Gα1 with GDP and magnesium fluorooxaluminate, whereas the K180P mutation destabilizes this state. The crystal structures of (K180P)Gα1 bound to a slowly hydrolyzable GTP analog, and the GDP-magnesium fluorooxaluminate complex provide evidence that the Mg²⁺ binding site is destabilized and that Switch I is torsionally restrained by the K180P mutation. The data are consistent with a catalytic mechanism for Gα in which major conformational transitions in Switch I and Switch II are obligate events that precede the bond-breaking step in GTP hydrolysis. In (K180P)Gα1, the two events are decoupled kinetically, whereas in the native protein they are concerted.

Heterotrimeric G proteins are activated by agonist-stimulated G protein-coupled receptors that catalyze the exchange of Mg²⁺GTP for GDP on G protein α (Gα) subunits. Upon binding GTP, Gα subunits dissociate from Gβγ heterodimers (1, 2), interact with effector proteins, and thereby control intracellular pathways. Thus, Gαi and Gαq, 2 of the 16 mammalian Gα isoforms, respectively stimulate and inhibit the catalytic activity of certain isoforms of adenylyl cyclase. However, activation is transient because Gα possesses intrinsic GTPase activity that restores it to the deactivated, GDP-bound state within 10–20 s at 30°C. Inactivation occurs because GTP hydrolysis induces conformational changes in the catalytic and effector-binding sites of Gα, which includes two polypeptide segments, called Switch I and Switch II. As a consequence of this transition, the affinity of Gα for effector is reduced whereas the affinity for Gβγ is increased, thereby terminating the cycle of signal transduction. Deactivation can be accelerated by GTPase-activating proteins, which include the regulators of G protein signaling (RGS) proteins (3).

The steady-state rate of Gα-catalyzed GTP hydrolysis is limited by the rate at which GDP is released from the enzyme. Consequently, pre-steady-state kinetic methods are used to measure the single-turnover rate at which GTP is converted to GDP. GTP hydrolysis is initiated by addition of the cofactor Mg²⁺ to Gα-GTP. Binding of Mg²⁺ induces a rapid increase in Trp fluorescence emission that reflects rapid conversion to the activated conformation. Fluorescence decays subsequently at a rate that parallels that of GTP hydrolysis: ∼2–4 min⁻¹ at physiological temperature (4, 5).

The crystal structure of Gα1 bound to GppNHp and the hydrolysis-resistant analog guanosine-5'-O-(β-pyridyl)triphosphate (GppNHP) shows that two critical catalytic residues, R178 in Switch I and Q204 in Switch II, adopt conformations that do not allow them to participate in catalysis (6, 7). However, when Gα binds the presumptive transition state analog GDP·Mg²⁺·AlF₄⁻, these residues and a segment of Switch I undergo a conformational rearrangement that affords their direct interaction with the pentacoordinate phosphoramide transition state (6, 8). Trp fluorescence emission is also enhanced in this state (9), and RGS proteins preferentially bind to this conformation of Gα (10–12). Thus, an active-site preceding step may occur before GTP hydrolysis can proceed. We refer to this preceding state as the pretransition state of Gα1.

The quenching of intrinsic fluorescence that accompanies GTP hydrolysis is attributed to a conformational change in Switch II in which W211 (W207 in transducin) (13) is transferred from a buried site in the enzyme to a solvent-exposed environment. This same conformational change may be responsible for effector release and Gβγ rebinding (14, 15). It is commonly believed that such conformational changes in G proteins occur as a consequence of GTP hydrolysis, although the two events appear to be concerted. Here, we suggest that, rather, these conformational changes are obligate steps in the reaction trajectory itself (Scheme 1). In Scheme 1, Q represents one or more GTP-bound, but low-fluorescence, conformational states that the enzyme must assume before the product complex is formed.

From analysis of Gα1, crystal structures, we inferred earlier that a substantial conformational change in Switch II is concomitant with or precedes the formation of the transient Gα1·GDP·P₂· ternary complex, and we have suggested that this conformational change might be rate-limiting (16, 17).

In the crystal structures of Gαi, Gαq, and Gα1 bound to GTP analogs, Switch II folds into an irregular helix (6, 18, 19) that, at its N terminus, makes hydrogen bond contact with the γ phosphate moiety of the nucleoside triphosphate. We reasoned that, if conformational changes in Switch II are required for catalysis, then mutations that either stabilize or destabilize its structure could affect the single-turnover rate of GTP hydrolysis. Accordingly, we conducted an Ala scan of residues in Switch II and mutated one residue in Switch I. The side chains of the residues that were mutated do not make direct contacts with the guanine nucleotide or Mg²⁺ in crystal structures of Gα1. Here, we present a kinetic, thermodynamic, and structural analysis of the catalytic properties of two of the Gα1 mutants that were produced in this study. The properties of these molecules provide insight into the relationship between conformational change and GTP hydrolysis in G proteins.

Materials and Methods

Purification of Gαi1, Gαi1 Mutants, and RGS4. Nonmyristoylated Gαi1 (20) and 6His-tagged RGS4 (21) were expressed in Escherichia coli

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Abbreviations: Gα, G protein α; RGS, regulators of G protein signaling; GppNHp, guanosine-5’-O-(β-pyridyl)triphosphate; GNF, GppNHP·Mg²⁺; GDP·AlF₄, GDP·Mg²⁺·AlF₄⁻; GTP·i, guanosine-5’-O-3-thiotriphosphate; MANT, N-methylanthraniloyl; mGTP, 2’(3’)-O-(N-methylanthraniloyl)-guanosine-5’-triphosphate.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes 1SVK and 1SVS for (K180P)Gα1·GDP·Mg²⁺·AlF₄⁻ and (K180P)Gα1·GppNHP·Mg²⁺, respectively].

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and purified as described. Mutants of Go11 were generated by using the QuikChange kit (Stratagene).

**Single-Turnover GTase Assays.** Single-turnover GTase assays were performed at 4°C, as described (21). We bound γ-32P-GTP (1 μM, 7,000 cpm/μmol) to Go11 (10 nM) in HEDL buffer (50 mM Hepes/1 mM EDTA/1 mM DTT/20 ppm of C12E8, pH 8.0) for 15 min at 30°C. Manually run assays were initiated by addition of 25 mM MgSO4 and 200 μM guanosine-5’-O-3-thiotriphosphate (GTPγS), with or without 2–3 mM RGS4. Reactions were quenched in a slurry of 15% charcoal in 50 mM H3PO4 (pH 2.3) at 0°C. Determination of [32P]Pi in the supernatant by scintillation counting. We fit the first-order exponential equations to derive rate constants by excitation at 366 nm and emission at 440 nm. The time courses were carried out for 5 and 20 min, respectively, to quenching rates, reactions were followed for 5 min by using an LS50B spectrophotometer (Perkin–Elmer).

**Stopped-Flow Fluorescence Single-Turnover Assays of GTP and 2’ (3’)-O-(N-Methylanthraniloyl)-Guanosine-5’-Triphosphate (mGTP) Hydrolysis.** The decrease in intrinsic Trp fluorescence of Go11 during hydrolysis of bound GTP, or in N-methyl-anthranoyl (MANT) fluorescence during hydrolysis of mGTP, were measured by using either a SLM4 (Biologic) or an SX185-MR (Applied Photophysics, Surrey, U.K.) stopped-flow instrument, as described (22). Proteins (500 nM) were preloaded with 2.5 μM GTP or 5.2 μM mGTP in HEDL buffer for 15 min at 30°C. GTP/mGTP-loaded Go11. Reactions were initiated by 30 mM MgSO4 in the presence of RGS4 (final concentration 160 μM). Trp fluorescence at 340 nm was excited at 290 nm. MANT fluorescence was followed by excitation at 360 nm and emission at 440 nm. The time dependence of Trp and MANT fluorescence emission were fit to first-order exponential equations to derive rate constants kW and kMANT, respectively. For wild-type Go11 and mutants with low Trp quenching rates, reactions were followed for 5 min by using an LS50B spectrophotometer.

**Stopped-Flow Single-Turnover Assay of Phosphate Release from Go11-GDP·P,**. The rate of release of free P, from the Go11-GDP·P complex was determined by using the EnzCheck assay kit (Molecular Probes) as described (23). Go11 (50 μM) in Mg2+-free HEDL buffer was incubated with 10 μM GTP in the presence or absence of RGS4 (16 μM) for 15 min at 30°C and then passed through a gel-filtration spin column to remove excess GTP. Assays were initiated by addition of 250 mM MgSO4/200 μM GTP/S/0.35 mM 2-amino-6-mercapto-methyluridine riboside/15 units/ml−1 purine nucleoside phosphorylase in a stopped-flow apparatus (Biologic). The increase in absorbance at 355 nm was fit to a single-order exponential equation to yield the rate constant kpi.

**Analysis of Reaction Models.** Progress curves for the consumption of Go11-GTP-Mg2+ (T) and the formation of Go11-GDP-Mg2+ (D), were modeled by using the mechanism shown in Scheme 2.

\[ \frac{d[T]}{dt} = -(k_1 + k_3)[T] + k_4[T] + k_2[Q]. \]

Concentrations of these species are normalized to 1.0 in arbitrary units, and initial conditions are set at [T]0 = 1.0 and [D]0 = 0.0. Progress curves for T, D, and Q are taken from Trp quenching of Go11-GTP (modeled with rate constant kW) and 32P production (rate constant kPi) upon addition of Mg2+·Q. The GT-bound Go11 species in which Trp fluorescence is quenched, cannot be observed directly, and its concentration is derived from the following conservation relation: [Q] = 1 – [T] – [D]. Values for k1, k−1, k2, and k3 in Scheme 2 were determined by using simplex and least-squares algorithms implemented in the computer program V2.0 (MicroMath Scientific, St. Louis).

**Isothermal Titration Calorimetry (ITC).** Experiments were performed by using a VP microcalorimeter (MicroCal, Amherst, MA). A typical titration involved 15–20 injections at 3-min intervals of 8-μl aliquots of RGS4 (450 μM in 50 mM Tris/1 mM EDTA/2 mM DTT/3 mM MgSO4, pH 8.0) into 1.344 ml of wild-type or mutant Go11 (50 μM) in the same buffer. For experiments that involved binding of Go11-GDP-Mg2+-AlF3 complexes to RGS4, the buffer also contained 5 μM GDP, 16 mM NaF, and 40 μM AlCl3. The sample cell was stirred at 400 rpm. The heats of dilution of the RGS4 in the buffer alone were subtracted from the titration data. The ITC binding data were fit to a single-site binding model by using ORIGIN software (Microcal) to determine the association constant Ks, thermodynamic parameters, and standard errors of the measurements for these values, as described (24).

**Crystallization and Structure Determination.** Crystals of K180PGo11 complexes were produced by the hanging-drop method. For crystallization of the complex containing GDP-Mg2+-AlF3 (GDP-AlF), 3 μl of 10–15 mg/ml protein in 20 mM Heps buffer containing 1 mM EDTA, 2 mM DTT, 5 μM GDP, 16 mM MgCl2, 16 mM NaF, and 40 μM AlCl3 was mixed with equal amounts of 2.1 M ammonium sulfate in 0.1 M sodium acetate.

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**Table 1. Pre-steady-state rates for GTP hydrolysis and conformational change for wild-type and mutant Go11 in the presence and absence of RGS4**

<table>
<thead>
<tr>
<th>Protein</th>
<th>k_{hyd} min⁻¹</th>
<th>k_W min⁻¹</th>
<th>k_{w} min⁻¹</th>
<th>k_{MANT} min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Go11</td>
<td>0.46 (0.05)</td>
<td>0.21 (0.03)</td>
<td>0.30 (0.02)</td>
<td>0.52 (0.03)</td>
</tr>
<tr>
<td>(G202A)Go11</td>
<td>4.2 (0.1)</td>
<td>3.1 (0.2)</td>
<td>4.9 (0.1)</td>
<td>4.9 (0.2)</td>
</tr>
<tr>
<td>(K180PGo11)</td>
<td>0.06 (0.01)</td>
<td>0.03 (0.01)</td>
<td>3.6 (0.6)</td>
<td>0.07 (0.01)</td>
</tr>
<tr>
<td>(K180AGo11)</td>
<td>0.31 (0.02)</td>
<td>0.22 (0.05)</td>
<td>0.25 (0.3)</td>
<td>0.41 (0.01)</td>
</tr>
<tr>
<td>(G202A/K180P)Go11</td>
<td>4.8 (0.6)</td>
<td>2.9 (0.6)</td>
<td>5.5 (0.3)</td>
<td>5.1 (0.4)</td>
</tr>
<tr>
<td>Go11·GDP</td>
<td>5.2 (0.6)</td>
<td>4.2 (0.6)</td>
<td>5.5 (0.8)</td>
<td>5.2 (0.4)</td>
</tr>
<tr>
<td>(G202A)Go11·G54</td>
<td>5.7 (0.6)</td>
<td>6.9 (0.2)</td>
<td>8.5 (0.2)</td>
<td>5.4 (0.2)</td>
</tr>
<tr>
<td>(K180P)Go11·G54</td>
<td>7.6 (0.5)</td>
<td>6.2 (0.5)</td>
<td>7.2 (1.1)</td>
<td>6.1 (0.8)</td>
</tr>
</tbody>
</table>

k_{hyd} is the first-order rate constant for GTP hydrolysis, as measured by acid quench of the GTase reaction. k_W is the rate constant for release of P, from the Go11-GDP·P, as measured by a coupled-enzyme reaction, which measures the generation of free phosphate. k_{MANT} and k_W are the rate constants for the decrease in fluorescence of bound MANT-GDP and Trp, respectively. Assays were conducted at 0°C to determine k_{hyd} and at 4°C for determination of k_{MANT}, k_W, and k_{w}. Kinetic parameters listed are average values determined from three experiments. Standard errors of measurement are given in parentheses.
reservoir buffer and set up as hanging drops. Crystals were cryo-
protected in reservoir buffer containing 15% (vol/vol) glycerol.
Crystals containing Gpp(NH)pMg\(^{2+}\) (GNP) were prepared as de-
scribed (7). Monochromatic x-ray data were measured at beam line
5.0.1 at the Advanced Light Source (Lawrence Berkeley National
Laboratory, Berkeley, CA) for crystals of (K180P)G\(_{\alpha i1}\)GDP-AIF
and at the beam line BM-19 at the Advanced Photon Source
(Argonne National Laboratory, Argonne, IL) for crystals of (K180P)G\(_{\alpha i1}\)GTP. Data sets extended to 1.5 and 2.0 Å for the
Gpp(NH)p- and GDP-AIF\(_{\alpha i1}\)-containing crystals. Data sets were
measured by the oscillation method in 0.5° frames and processed by
using the HKL 2000 package (HKL Research, Charlottesville, VA)
(25). Structures were determined by molecular replacement by
using the 1GFI and 1CIP coordinate sets as starting models. Atomic
models were refined by using the CNS 1.1 program package (26),
withholding 5% of the data for computation of \(R\_\text{free}\). Parameter
files from Engh and Huber (27) were used for protein atoms; files
for AIF\(_{\alpha i1}^2\), GTP, and GDP were obtained from the Hetero-
compound Information Centre, Uppsala (28). Atomic models were
refit into \(\sigma\)-weighted \(F_i - F_o\) electron-density maps by using the
program O (29).

**Difference-Distance Computations.** Calculations were carried out by
using a modified version of the Difference Distance Matrix Plot
(DDMP) program (Center for Structural Biology, Yale University,
New Haven, CT).

For additional information regarding experimental methods, see
**Supporting Materials and Methods**, which is published as supporting
information on the PNAS web site.

**Results**

**Mutational Acceleration and Decoupling of GTPase Activity.** We
constructed a set of G\(_{\alpha i1}\) mutants in which Switch II residues
202–212 (excluding 203 and 204), were individually replaced by Ala.
Also, proteins were created in which K180 in Switch I was replaced
with Pro, its cognate in the sequence form G\(_{\alpha i1}\) (30), or with Ala. The
K180P/G202A double mutant was constructed also. All of the
recombinant mutant proteins were produced in E. coli at levels comparable with that of the wild-type protein. The guanine nucleo-
tide-exchange activity of each mutant was determined by a filter-
binding assay using \([\text{35S}]\text{-labeled GTP}\)S, and the GTPase activity
of the mutants was determined by a single-turnover assay using
\([\gamma^3P]\text{GTP} (20). The first-order rate constants for nucleotide
exchange and GTP hydrolysis for most of the mutants were similar
to that of wild-type G\(_{\alpha i1}\), and these proteins were not studied
further. In contrast, the hydrolysis rates measured for the (K180P),
(G202A), and (K180P and G202A) mutants differed substantially from
that of the wild type.

Events triggered by Mg\(^{2+}\) activation of the GTP complexes of the
mutant G\(_{\alpha i1}\) proteins were followed by four different pre-steady-
state assays conducted in the presence and absence of the GTPase
activator RGS4. These results of these experiments are summarized
in Table 1.

The single-turnover rate of GTP hydrolysis from G\(_{\alpha i1}\)G\(_{\alpha i1}\)GDP
is measured by the \(32\text{P}\)-labeled G\(_{\alpha i1}\)GDP-P released after ac-id-quench of the reaction
at various time points after addition of Mg\(^{2+}\) (Fig. 1A). Because
G\(_{\alpha i1}\) is denatured by the quench, the quantity of \(32\text{P}\)-labeled GDP-P measured in the
assay reflects its rate of production rather than the rate at which
it dissociates from the G\(_{\alpha i1}\)GDP-P complex. In all cases, the
evolution of phosphate could be fit to a first-order rate equation,
characterized by the rate constant \(k_{\text{hyd}}\) (Table 1). Analysis of data
taken at 100-ms intervals within the first 2 s of the reaction phase
showed no evidence of a burst-phase for any of the G\(_{\alpha i1}\) proteins
(data not shown). The GTPase rate constant for (G202A)G\(_{\alpha i1}\) is
~10-fold greater than that of the wild-type protein, whereas the
K180P mutation, as had been shown earlier (30), reduced \(k_{\text{hyd}}\) to
<15% that of wild-type G\(_{\alpha i1}\).

That mutations of either of these residues might alter the rate of
GTP hydrolysis is not surprising. The main-chain amide groups that
follow both residues in sequence form hydrogen bonds to the \(\gamma\)
phosphate of GppNHp (7). In the GDP-AIF complex of G\(_{\alpha i1}\), the
\(\zeta\) amino group of K180 lies within hydrogen bond distance of the
axial hydroxyl ligand of AIF\(_{\alpha i1}^2\). This hydroxyl group mimics the
nucleophile in GTP hydrolysis, and therefore, it might be inferred
that K180 participates in the catalytic mechanism. However, \(k_{\text{hyd}}\) for
(K180A)G\(_{\alpha i1}\) is similar to that of wild-type G\(_{\alpha i1}\) (Table 1 and Fig.
1A), suggesting that the effect of the K→P mutation is not due to
the loss of the Lys side chain but rather to its substitution by a
constrained pyrrolidine ring. If both K180P and G202A mutations are
present, the latter has a fully dominant effect on the GTP
hydrolysis rate. The GTPase activity of recombinant (K180P/
G202A)G\(_{\alpha i1}\) is similar to that of the G202A mutant.

In wild-type G\(_{\alpha i1}\), Mg\(^{2+}\) binding is accompanied by a rapid
increase in fluorescence at 340 nm, which is attributed to a change
in the environment of the Trp in Switch II. Fluorescence subsequently
decays in conjunction with GTP hydrolysis such that the
first-order rate constants for the two processes are the same.
\(k_{\text{hyd}}\) and \(k_{W}\) are also nearly equal for the G202A mutant and
~10-fold greater than the corresponding rate constants for the
wild-type protein (Fig. 1B and Table 1). In contrast, the Trp
quenching rate of (K180P)G\(_{\alpha i1}\) is similar to that of the G202A
mutant, even though (K180P)G\(_{\alpha i1}\) hydrolyzes GTP at only 15% of
the wild-type rate. The K180P mutation effectively decouples the
transformations associated with GTP hydrolysis and the perturba-
tion of the Trp in Switch II. For the K180P/G202A double mutant,
Subsequent to GTP hydrolysis, inorganic phosphate is released rapidly from the wild-type Go_{11}GDP-P\_i complex. Phosphate release is monitored by a fast-coupled enzyme reaction that produces a fluorescent product (23) (see Fig. 4, which is published as supporting information on the PNAS web site). For Go_{11} and its mutants, product release fits a first-order rate equation with no apparent lag phase (Table 1). Rate constants $k_{pi}$ for all proteins, in experiments conducted in the absence of RGS4, are 50–70% of their corresponding values of $k_{pi}$. The differential could in part reflect insensitivity of the coupled enzyme reaction to low $P_i$ concentration. However, $k_{pi}$ for (K180P/G202A)Go_{11}, which possesses a high GTPase rate, is also one-half of that of $k_{pi}$ and consistent with a short but detectable lifetime for the tertiary GDP-P\_i-enzyme complex. In the presence of RGS4, the rate of phosphate release does not differ significantly from that of hydrolysis.

The single-turnover kinetics of the GTPase reaction can also be measured by the rate at which MANT fluorescence of mGTP is quenched upon the release of weakly bound mGDP from Go_{11} (31). For Go_{11} and all mutants, the apparent first-order rate constant for fluorescence decay ($k_{MANT}$) was found to be within 25% of $k_{pi}$ (Table 1 and see Fig. 5, which is published as supporting information on the PNAS web site).

The effects of RGS4 on the K180P mutant were similar to those observed (30), and for G202A and the K180P/G202A double mutants, acceleration of GTP hydrolysis and fluorescence decay were relatively minor because the basal activities of these mutants are so high. The affinity measurements described below are more informative of the interactions of these proteins with RGS4.

**K180P Perturbs the Structure of the Pretransition State for GTP Hydrolysis.** To understand the structural basis for the effects of the K180P and G202A mutations, we set out to determine the structures of the mutant proteins bound to GNP and GDP-AIF. Attempts to crystallize (G202A)Go_{11} were unsuccessful, but the K180P mutant could be readily crystallized. The structures of the GNP and GDP-AIF complexes of (K180P)Go_{11} were determined at resolutions of 1.5 and 2.0 \AA and refined to $R_{free}$ values of 0.21 and 0.23, respectively (Table 3, which is published as supporting information on the PNAS web site).

Relative to the respective structures of wild-type Go_{11} (determined at 1.5- and 2.2-\AA resolution), the rms deviations in the positions of corresponding main chain atoms are only 0.18 and 0.27 \AA, indicating the absence of substantial global changes in structure.

Structural differences at the site of mutation and within the active site of (K180P)Go_{11} are clearly evident. The Pro substitution constrains the main-chain \(\phi\) and \(\psi\) angles of residue 180 to similar values (within 2° for both angles) in the GNP and GDP-AIF states, whereas in wild-type Go_{11}, \(\phi\) and \(\psi\) both decrease by \(\approx5–7°\) in the transition from the former state to the latter state. The main-chain angles at P180 for the GDP-AIF complex are more similar to those of the GNP complex of the wild-type protein than they are to the GDP-AIF complex. Thus, substitution of Pro for Lys both drives the local conformation of Switch I toward the ground (GNP) state and appears to impose a torsional constraint.

The Mg$^{2+}$ binding site of Go_{11} is also altered in the GDP-AIF Complex (Fig. 2). S47, a direct Mg$^{2+}$ ligand, shows static disorder (see Fig. 6, which is published as supporting information on the PNAS web site). Asp-200, a second-shell ligand that is highly conserved in the Ras superfamily, adopts a different side-chain conformation than that observed in the wild-type protein. As a consequence of these changes, a cooperative hydrogen bond network involving Mg$^{2+}$, one of two water ligands, together with D200 and S47, is partially disrupted. No comparable differences are apparent between the GNP complex of (K180P)Go_{11} and that of the wild type.

At a more global level, it is evident that the K180P mutation has different structural consequences in the GNP and GDP-AIF-bound states. The latter is manifested in the changes in inter-Co distances induced by the K180P mutation (i.e., \(\Delta Dij\), the change in the distance between the Co atoms of residue \(i\) and residue \(j\) in (K180P)Go_{11} relative to that of the wild type; Fig. 3). The magnitudes of interresidue changes are typically small (<0.7 Å) but clearly segmental. Among residues within a 10-Å radius of residue 180, the P-loop (residues 40–50), Switch I (residues 166–183), and Switch II (residues 200–213) are particularly altered (Fig. 3 A and B). The magnitudes of the interresidue shifts due to the transformation from the GNP-bound to the GDP-AIF-bound states differ in the wild-type and P180 backgrounds. This apparent nonadditivity of the two perturbations (mutation vs. change in ligand) is evident in Fig. 3C, in which the changes in pairwise Co–Co distances due to the K180P mutation in the GNP-bound proteins are subtracted from the corresponding distance changes in the GDP-AIF-bound proteins (i.e., \(\Delta Dij^\text{GNP} - \Delta Dij^\text{AIF}\)). It is reasonable to conclude that the K180P mutation has specific effects on the transformation of the ground state of Go_{11} to the pretransition state represented by the GDP-AIF complex.
whereas the K180P mutation perturbs this state. Nevertheless, 
the bound G state might be reflected in a change in its affinity for RGS4.
atic of changes in the structure of the ground state.
GTPase activity in the presence of RGS4 (Table 1). Posner 
et al. form of (K180P)G
7564
Hydrolysis.
Because RGS4 binds preferentially to the GDP
AlF complex of (K180P)G
i1
AlF4
GppNHp
GDP
AlF
GDP
AlF
GTP
GTP
Pi
Q
Switch II
k1
k2
k3
Scheme 2.

As measured by isothermal titration calorimetry (Table 2), the 
the GDP-bound complexes (Left), and the AlF-bound complexes (Center) are shown. In Right, the elements from the AlF matrix are subtracted from the corresponding elements in the GNP matrix. Values are r-weighted and color-coded according to direction and magnitude (red, negative; blue, positive). Contour values range from ±σ to 0. Matrix elements corresponding to residue pairs separated by >10 Å were set at 0. The dark line represents self-vectors (i = j).

Fig. 3. Difference-distance analysis of wild type and (K180P)Goαi1 in complexes with GppNHp-Mg2⁺ and GDP-Mg2⁺-AlF4. Changes in contacts between Ca in residues 165–207 in Goαi1 (rows) and residues 35–76 and 140–226 in (K180P)Goαi1 (columns) for the GNP-bound complexes (Left), and the AlF-bound complexes (Center) are shown. In Right, the elements from the AlF matrix are subtracted from the corresponding elements in the GNP matrix. Values are r-weighted and color-coded according to direction and magnitude (red, negative; blue, positive). Contour values range from ±σ to 0. Matrix elements corresponding to residue pairs separated by >10 Å were set at 0. The dark line represents self-vectors (i = j).

Table 2. Affinity of RGS4 for nucleotide-bound complexes of wild-type and mutant Goαi1

<table>
<thead>
<tr>
<th>Protein</th>
<th>GDP</th>
<th>GppNHp</th>
<th>GDP-AlF⁺</th>
<th>Ratio of GppNHp/GDP-AlF⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goαi1</td>
<td>430</td>
<td>5.2 (0.9)</td>
<td>0.13 (0.1)</td>
<td>43</td>
</tr>
<tr>
<td>(G202A)Goαi1</td>
<td>18</td>
<td>1.4 (0.1)</td>
<td>0.13 (0.8)</td>
<td>11</td>
</tr>
<tr>
<td>(K180P)Goαi1</td>
<td>670</td>
<td>2.3 (0.5)</td>
<td>1.1 (0.1)</td>
<td>2</td>
</tr>
</tbody>
</table>

Data are expressed as Kd (in μM). Standard errors of measurement are given in parentheses.

G202A Stabilizes and K180P Perturbs the Pretransition State for GTP Hydrolysis. Evidence that the K180P mutation differentially perturbs the ground and pretransition states of Goαi1 led us to investigate the affinity of RGS4 for the GNP-bound and GDP-AlF-bound complexes of the G202A and K180P mutants of Goαi1. Because RGS4 binds preferentially to the GDP-AlF complex of Goαi1 (10), mutation-induced perturbation of the structure of this state might be reflected in a change in its affinity for RGS4. Similarly, differences in the affinity of RGS4 for GDP- or GNP-bound Goαi1 mutants relative to the wild-type complexes are indicative of changes in the structure of the ground state.

As measured by isothermal titration calorimetry (Table 2), the GNP complexes of (K180P)Goαi1 and (G202A)Goαi1 are similar to that of wild-type Goαi1 in their affinity for RGS4. The GDP-bound form of (K180P)Goαi1, like the wild-type protein, has low affinity for RGS4, whereas (G202A)Goαi1-GDP binds more tightly. Last, the GDP-AlF complex of (K180P)Goαi1 has less affinity for RGS4 than (G202A)Goαi1 or the wild-type protein. Therefore, it appears that the G202A mutation stabilizes the pretransition state of Goαi1, whereas the K180P mutation perturbs this state. Nevertheless, (K180P)Goαi1, (G202A)Goαi1, and Goαi1 all attain similar levels of GTPase activity in the presence of RGS4 (Table 1). Posnet et al. (30) found that the EC50 of RGS4 for stimulation of the steady-state GTPase activity of (K180P)Goαi1 in the presence of M2 receptors is 10-fold higher than that for wild-type Goαi1, but its efficacy for the two Goα proteins is the same.

Discussion
For Goαi1, GTP hydrolysis and conformational change are seemingly concerted. Single-turnover assays designed to measure the rate of GTP hydrolysis, product (P) release, and conformational change in Switch II yield progress curves that can be fit with similar first-order rate constants. In the presence of RGS4, all three events are apparently accelerated by the same factor. Release of P from the Goαi1-GDP-P, occurs too rapidly to be distinguished accurately from the rate at which it is produced within the enzyme. Less apparent is the relationship between the reaction trajectory for GTP hydrolysis and the conformational change that is signaled by decay of the Mg2⁺-induced fluorescence of W211.

Mutation of G202 to Ala increases the turnover rate for GTP hydrolysis ~10-fold. We have not succeeded in crystallizing this mutant and, therefore, can offer only a speculative rationale for its effect on GTP hydrolysis. In a model of (G202A)Goαi1-GTP based on the crystal structure of the wild-type protein, steric conflict arises from a 2-Å contact between the methyl side chain of A202 and the carbonyl oxygen atom of T181. This distance increases to 2.5 Å in the model of the (G202A)Goαi1 complex with GDP-AlF. By destabilizing the ground state (i.e., Goαi1-Mg2⁺-GTP), the G202A mutation may promote the structural transition to the pretransition state that is mimicked by the GDP-AlF complex. Accordingly, the selectivity of RGS4 for the Goαi1-GDP-AlF complex increases 4-fold in the background of the G202A mutation (Table 2).

The kinetic behavior of the K180P mutant suggests that otherwise concerted events in Goα-catalyzed GTP hydrolysis can be disengaged. That Trp quenching occurs 50-fold faster than GTP hydrolysis indicates that two distinct species arise after addition of Mg2⁺ to the Goαi1-GTP complex as indicated in the kinetic mechanism shown in Scheme 2. In this mechanism, species Q corresponds to GTP-bound (K180P)Goαi1 in which intrinsic Trp fluorescence is quenched, and Goα⁺GTP is the Mg2⁺-activated complex with high intrinsic Trp fluorescence. Breakdown of Goαi1-GDP-P, is considerably more favorable than formation, as indicated by insensitivity of the enzyme to inhibition by phosphate (32).

The rate constants kω and kω0 (Table 1) are first-order approximations to the progress curves for the disappearance of Goα⁺GTP (Fig. 1B) and appearance of Goα GDP-P (Fig. 1A), respectively. Because state Q is not directly observable, the progress curves cannot be used to derive a unique set of values for all four constants in Scheme 2. However, the progress curves do place severe limits on the values of these rate parameters. Q must lie on a catalytically productive pathway because models in which the value of k2 is constrained to zero give poor fits to the progress curves. Such models produce clearly biphasic time courses. Further, a model in which k3 is constrained to zero converges well to a solution with k1 = 3.9 min⁻¹, k⁻¹ = 0, and k2 = 0.33 min⁻¹. The SEMs of these fits to the experimental data are less than the standard errors of measurement. The kinetic parameters so derived point to a simple,
irreversible reaction: $\text{T} \rightarrow \text{O} \rightarrow \text{D}$, in which $\text{Q}$ is an obligate reaction intermediate. Unconstrained fitting of all four parameters produces values of $k_1$ and $k_2$ similar to those presented above, but $k_3$ and $k_5$ converge to 0.28 min$^{-1}$ and 0.19 min$^{-1}$, respectively. Although conversion of Q to GDP-Pi, modeled as an irreversible step, the kinetic data presented here do not rule out the possibility of rapid interconversion of the two species, as has been considered for GTPase-activating protein-stimulated Ras (33).

The intermediate, Q, that is proposed to arise in the GTPase reaction catalyzed by (K180P)$\text{G}_{\text{i1}}$, must adopt a conformation in which the fluorescence of Trp-211 is substantially quenched but is position to orient a water molecule for nucleophilic attack on the Q state because the catalytic Gln in these structures is not in position to orient a water molecule for nucleophilic attack on the Quaternary (Q) state. We infer that similar intermediate conformational changes appear to be an obligate step in catalysis rather than a consequence of the reaction. We thank staff at the Structural Biology Center, Advanced Photon Source (Argonne National Laboratory, Argonne, IL), and Advanced Light Source (Lawrence Berkeley National Laboratory, Berkeley, CA) for assistance with data collection. We especially thank Craig Doupnik and Qing Li Zhang for sharing their data with us before publication, and Arne Strand for his assistance with the kinetic-modeling calculations.

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