The A326S Mutant of $G_{iai1}$ as an Approximation of the Receptor-bound State*

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Bruce A. Posner‡, Mark B. Mixon§, Mark A. Wall§, Stephen R. Sprang§, and Alfred G. Gilman¶

From the Departments of ¶Pharmacology and §§Biochemistry and the ¶Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9041

Agonist-bound heptahelical receptors activate heterotrimeric G proteins by catalyzing exchange of GDP for GTP on their $\alpha$ subunits. In search of an approximation of the receptor-$\alpha$ subunit complex, we have considered the properties of A326S Gi, a mutation discovered originally in $G_{iai}$ (Iiri, T., Herzmark, P., Nakamoto, J. M., Van Dop, C., and Bourne, H. R. (1994) Nature 371, 164–168) that mimics the effect of receptor on nucleotide exchange. The mutation accelerates dissociation of GDP from the $\alpha_1 \beta_2 \gamma_2$ heterotrimer by 250-fold. Nevertheless, affinity of mutant $G_{iai}$ for GTP$\gamma$S is high in the presence of Mg$^{2+}$, and the mutation has no effect on the intrinsic GTPase activity of the $\alpha$ subunit. The mutation also uncouples two activities of $\beta\gamma$: stabilization of the GDP-bound $\alpha$ subunit (which is retained) and retardation of GDP dissociation from the heterotrimer (which is lost). For wild-type and mutant $G_{iai}$, $\beta\gamma$ prevents irreversible inactivation of the $\alpha$ subunit at 30 °C. However, the mutation accelerates irreversible inactivation of $\alpha$ at 37 °C despite the presence of $\beta\gamma$. Structurally, the mutation weakens affinity for GTP$\gamma$S by steric crowding: a 2-fold increase in the number of close contacts between the protein and the purine ring of the nucleotide. By contrast, we observe no differences in structure at the GDP binding site between wild-type heterotrimers and those containing A326S Gi. However, the GDP binding site is only partially occupied in crystals of G protein heterotrimers containing A326S Gi. In contrast to original speculations about the structural correlates of receptor-catalyzed nucleotide exchange, rapid dissociation of GDP can be observed in the absence of substantial structural alteration of a $G_{iai}$ subunit in the GDP-bound state.

Heterotrimeric guanine nucleotide-binding proteins (G proteins) function as molecular switches during receptor-stimulated signal transduction (1–3). G proteins are activated by agonist-bound heptahelical receptors, and they subsequently modulate second messenger synthesis and other processes by interacting with downstream effectors. Activation results from receptor-catalyzed exchange of GDP for GTP on the G protein $\alpha$ subunit and dissociation of GTP-$\alpha$ from a high affinity dimer of $\beta$ and $\gamma$ subunits. Hydrolysis of bound GTP by $\alpha$ causes deactivation and reassociation of GDP-$\alpha$ with $\beta\gamma$.

X-ray crystallographic visualization of G protein-mediated signal transduction has been one of our primary goals, and high resolution structures of several intermediates in these cycles have been obtained by us and others (for review, see Ref. 3). However, the structures of a G protein-coupled receptor and a receptor-G protein complex remain unsolved and will be major and difficult accomplishments. It is thus of interest to characterize G protein mutants that may mimic the effect of receptor on nucleotide exchange. Iiri et al. (4) first characterized the A366S mutant of $G_{iai}$, the G protein $\alpha$ subunit responsible for activation of adenylyl cyclase. Ala-366 in $G_{ia}$ is in a loop connecting $\beta6$ and $\delta5$ in the Ras-like domain of the $\alpha$ subunit. The residues of this loop interact with and stabilize the guanine ring of GDP or GTP. The A366S mutation was discovered in male patients exhibiting an unusual combination of type Ia pseudohypoparathyroidism and gonadotropin-independent precocious puberty or testotoxicosis. Testotoxicosis in these patients results from receptor-independent activation of testicular adenylyl cyclase, caused by accelerated exchange of GDP for GTP; the mutation thus mimics the effect of receptor-mediated activation of $\alpha$. In addition, the mutant protein is temperature-sensitive, denaturing at normal body temperature but surviving in the relatively cool environment of the testes. The resultant deficiency of $G_{iai}$ in non-testicular tissues is responsible for the phenotype of pseudohypoparathyroidism.

Because accelerated dissociation of GDP from $G_{ia}$ proteins is a requisite characteristic of the receptor-G protein complex, we have evaluated in detail the biochemical and structural features of the corresponding mutation of $G_{iai}$ (A326S), a G protein $\alpha$ subunit that is particularly well suited to such analysis.

MATERIALS AND METHODS

DNA Manipulations—Mutagenesis of a $G_{iai}$ subunit was performed using the Bio-Rad Mutage kit. The mutagenic oligonucleotide used to construct A326S Gi, was 5'-CTCGTTATCCGAGCAAGTGAAAGT-3'. Manipulations and analyses of recombinant DNA were performed with standard protocols (5). The DNAs encoding all mutant proteins were completely sequenced.

Nucleotides, Proteins, and Buffers—GDP and GTP were purchased from Boehringer Mannheim. [$^{35}$S]GTP$\gamma$S and [$\gamma$-32P]GTP were purchased from NEN Life Science Products and purified using the method of Johnson and Walseth (6). [$\alpha$-32P]GDP was synthesized from [$\alpha$-32P]GTP and purified as described (6).

Mutant G protein $\alpha$ subunits were synthesized in Escherichia coli and purified as described previously, except that 30 mM AlCl$_3$, 10 mM NaF, 10 mM MgCl$_2$, 10% $\nu\nu$ glycerol, and 25 mM GDP were always

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The atomic coordinates and structure factors (code 1BH2) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9041.

§ The abbreviations are as follows: GTP$\gamma$S, guanosine 5'-[3-O-thio]-triphosphate; RGS4, regulator of G-protein signaling 4; $R$, $R$-factor = $\Sigma$|$F_u$ - $F_c$|/|$F_u$|; $R_{mean}$, the $R$-factor for 10% of the reflections in the data set that were excluded from refinement.
present to stabilize the protein (7, 8). Myristoylated proteins were utilized for all biochemical studies. Crystallization was achieved with nonmyristoylated protein with or without an amino-terminal hexahistidine tag. Large scale production and purification of βγγγG, C68S was accomplished using a baculovirus expression system, as described previously (9). Purified C68S and βγγγG were generous gifts from Berman and Alex Duncan, respectively (University of Texas Southwestern Medical Center, Dallas, TX). All proteins were homogeneous by SDS-polyacrylamide gel electrophoresis, and the amount of active mutant and wild-type α subunit was determined using a GTPγS binding assay (see below).

**Dissociation Rate Constants for Nucleotides**—Unless noted, all assays of nucleotide binding and hydrolysis were conducted at 30 °C in 50 mM NaHepes (pH 8.0), 1 mM EDTA, 3 mM dithiothreitol, and 0.05% polyoxyethylene 10-lauryl ether. The rates of dissociation of nucleotides from mutant and wild-type α subunits were measured by ligand competition (10). GTPγS was used as the competing ligand, and protein-bound [35S]GTPγS (800–1200 cpm/pmol) or [α-32P]GDP (800–1200 cpm/pmol) was detected with a filter-binding assay. Data were fit to a single exponential decay or a single exponential rise, depending on whether the bound ligand or competing ligand was radiolabeled, respectively. Curve fitting analysis was performed using SigmaPlot 4.17 for Macintosh.

**Nucleotide Hydrolysis**—Steady-state hydrolysis of GTP by mutant and wild-type α subunits was monitored by quenching the reaction with neutral charcoal (11, 12). To determine the apparent affinity ($K_a$) of the protein for Mgγ2+, the concentration of the divalent cation was varied as described previously, and the data were fit to Equation 1, where $V_a$ is the rate of the GTPase reaction at saturating Mgγ2+, $E_a$ is the total protein concentration, and $K_a$ is the concentration of Mgγ2+ that is not bound to EDTA. This equation takes into account the fact that protein-bound Mgγ2+ is a significant fraction of $E_a$.

$$ V = \frac{C \times V_a}{2} \times [E_a]^{1/2}, \quad \text{(Eq. 1)} $$

Single-turnover GTPase measurements were conducted using the same assay system. In these experiments, the α subunit (200 μM) was labeled with [γ-32P]GTP (700–2000 cpm/pmol) in the presence of 5 mM EDTA. The reaction was initiated by adding 10 mM MgCl2 and 100 μM GTP. Data were fit to a single exponential rise function.

**Protein Crystallization**—Crystals of A326S Gia1, complexed with GTPγS were grown using the hanging drop method. Crystals grew in 5–9 days, and several were stabilized for low temperature data collection using previously published methods (13).

The mutant heterotrimer was crystallized using a previously published protocol (9) with the following modifications. Unmodified A326S Gia1 (15 mg/ml) was mixed with βγγγG (18.7 mg/ml) in a final volume of 100 μl and incubated at 30 °C for 10 min. The mixture was then chromatographed over a Superdex G75 column (Amersham Pharmacia Biotech) in tandem with a Superdex G200 column in 50 mM NaHepes (pH 8.0), 1 mM EDTA, 3 mM dithiothreitol, and 150 mM NaCl. The heterotrimer was collected, concentrated to 15–20 mg/ml, and crystallized using the hanging drop method. Each drop (3 μl) consisted of 1.5 μl each of protein solution and well solution (18–25% polyethylene glycol 8000, 100 mM NaHepes (pH 7.0), 100 mM sodium acetate (pH 6.4), 0.05% n-β-octylglycylside, and 2% propanol). The drop was equilibrated with the well solution for 1–2 days before crystals appeared. Crystals were cryoprotected as described previously (9).

**Structural Studies**—Data sets were collected from single, cryoprotected crystals of the A326S Gia1, GTPγS-Mgγ2+ and A326S Gia1, GDP-βγP complexes using 0.908 Å radiation at the A1 beamline of the Cornell High Energy Synchrotron Source (CHESS) (Table I). Crystals were mounted with the $c^\alpha$ axis perpendicular to the beam and maintained at a constant temperature of 110 K in a liquid nitrogen-cooled air stream generated by an Oxford Cryosystem. Diffraction intensities were measured in the oscillation mode in 0.5° steps around the $\phi$ axis using a 2 K (2048 × 2048 pixels) charge-coupled device detector system set in the binned mode. With a 0.2-mm colimator, the mosaicity of the heterotrimer was approximately 0.5° and that for the α-GTPγS complex was 0.7°. Observed intensities were indexed and integrated with DENZO and merged and scaled using the HKL program suite (14).

Starting models for the refinement of A326S Gia1, GTPγS-Mgγ2+ and A326S Gia1, GDP-βγP were obtained by molecular replacement using the coordinates of the corresponding wild-type complexes (Protein Data Bank [Bernstein] identification codes 1GIA and 1662) as search models in the program AMORE (15). To avoid phase bias, all ligands were removed from the coordinate files. The correct rotation and translation solutions were readily identified. In both cases, the model was positioned at the appropriate coordinates in the unit cell and subjected to a single round of rigid body refinement.

For both complexes of A326S Gia1, a bulk solvent correction was made to the calculated structure factors. The scale and B-factors of the solvent models were calculated for the appropriate resolution range and in each case significantly improved the R-factor of the protein model in lower resolution shells. After an initial round of Powell minimization and restrained individual B-factor refinement in XPLOR 3.8 (16), the coordinates were subjected to simulated annealing at 1000 °C to help remove model bias introduced by molecular replacement (17). Convergence was achieved after cycles of manual model building, followed by Powell minimization and restrained B-factor refinement. Atomic models were refit to SigmaA-weighted $F_o-F_c$ and $F_c-F_o$ maps (18) using the program O (19). The free R-factor was used throughout to monitor the progress of refinement (16). Final refinement statistics are presented in Table II. The occupancy of GDP in crystals of heterotrimers formed with A326S Gia1 was determined as follows. A series of six coordinate files was created in which the B-factors of all nucleotide atoms were set to 40 Å2. The occupancy parameters for the nucleotide atoms were set to 1.0, 0.50, 0.38, 0.33, 0.25, and 0.12, respectively, for coordinate files 1 through 6. Restrained individual B-factors were then refined for all atoms in each of the six coordinate sets. The correct nucleotide occupancy was assumed to correspond to that which produced the lowest $R_{merge}$ and for which the average over nucleotide B-factors was nearest the average over the B-factors for the entire set. According to this criterion, the occupancy of GDP for this data set is approximately 0.33, for which $R = 0.254$, $R_{merge} = 0.269$, (B GDP) = 35 Å2, and (B GDP) = 47 Å2. In contrast, all converged parameter values using a GDP occupancy of 1.0 were $R = 0.282$, $R_{merge} = 0.291$, (B GDP) > 100 Å2, and (B GDP) = 40 Å2, and, for a GDP occupancy of 0.12, $R = 0.256$, $R_{merge} = 0.270$, (B GDP) = 4 Å2, and (B GDP) = 49 Å2.

**RESULTS**

**Dissociation Rate Constants for Nucleotides**—The dissociation rate constants ($k_{on}$) for GDP or GTPγS in binary or ternary complexes were determined using a competition assay (Scheme 1). In each assay, the Gia1 complex was diluted into a solution containing an excess of competing ligand (B), such that $k_1 << k_{B}[B] >> k_1[A]$. The extent of nucleotide exchange was monitored by detection of the radiolabeled complex of the ligand of interest (A = βγγγG [32P]GDP or [35S]GTPγS) or the competing ligand (B = [35S]GTPγS). Representative experiments are shown in Figs. 1 and 2, and the data are summarized in Table III.

Assuming the rates of nucleotide association are not affected by the mutation, we can conclude that the binding of GDP or GTPγS (in the absence of Mgγ2+) is weakened roughly 25- and 35-fold, respectively, relative to wild-type Gia1. These results are consistent with the proximity of the mutation to the guanine ring in the nucleotide binding pocket. $A1F_2$ binds to Gia1,GDP in a position normally occupied by the ψ-phosphate of GTP and activates the G protein α subunit.
in the presence of Mg$^{2+}$ (13, 20). AlF$_4^-$ and Mg$^{2+}$ slow the rate of GDP dissociation 10-fold for A326S G$_{i\alpha 1}$ but only 2-fold for the wild-type protein. Thus, the activation of regions surrounding the terminal phosphate and Mg$^{2+}$ binding sites appears to counteract most of the effect of the mutation. An even more dramatic effect is observed for GTP$\gamma$S binding in the presence of Mg$^{2+}$, which is essentially irreversible for both the mutant and wild-type proteins (Fig. 1).

There is positive cooperativity in the binding of GDP and $\beta\gamma$ to wild-type G$_{i\alpha 1}$. This interaction is an important aspect of the inactivation of dissociated G protein subunits and of stabilization of the heterotrimer. Specifically, $\beta\gamma$ binds to wild-type G$_{i\alpha 1}$ and slows dissociation of GDP roughly 6-fold. Although $\beta\gamma$ still forms a complex with the mutant $\alpha$ subunit, dissociation of GDP from A326S G$_{i\alpha 1}$ is actually facilitated modestly by $\beta\gamma$ (Fig. 2 and Table III). Subtle structural changes in the mutant heterotrimer have uncoupled the binding of $\beta\gamma$ from effects on dissociation of GDP (see below).

Nucleotide Hydrolysis—The GTPase activities of wild-type and A326S G$_{i\alpha 1}$ were estimated in single turnover assays. The $\alpha$ subunits were loaded with GTP in the absence of Mg$^{2+}$, and a single round of hydrolysis was initiated by addition of the divalent cation. The rates ($k_{\text{off}}$) for mutant and wild-type proteins are nearly identical (3.2 and 3.0/min, respectively; Fig. 3).

The rate of nucleotide dissociation ($k_{\text{off}}$) and the intrinsic rate of nucleotide hydrolysis ($k_{\text{cat}}$) predict the steady-state rate of nucleotide hydrolysis ($k_{\text{cat}}$).

$$k_{\text{cat}} = (k_{\text{cat}} \times k_{\text{off}})/(k_{\text{off}} + k_{\text{cat}}) \quad (\text{Eq. 3})$$

For the wild-type protein, the rate of GDP dissociation is slow ($k_{\text{off}} = 0.087$/min) and limits the measured steady-state rate of GTP hydrolysis ($k_{\text{cat}} = 0.062$/min). In the case of the mutant protein, both $k_{\text{off,GDP}} = 2.1$/min and $k_{\text{cat}} = 3.2$/min contribute to the steady-state rate. The predicted $k_{\text{cat}}$ of 1.26/min agrees well with the observed rate of 1.3/min and indicates that $\alpha$-GTP-Mg$^{2+}$ accumulates at steady state to a level of 40% of the total protein. To confirm these results, we conducted steady-state assays in the presence of the GTPase-activating protein RGS4 (Fig. 4). Under these conditions, $k_{\text{cat}}$ is increased more than 40-fold (data not shown) and $k_{\text{off}}$ for GDP now limits the steady-state hydrolytic rate as predicted ($k_{\text{cat,1}} = 2.2$/min; $k_{\text{off,GDP}} = 2.1$/min). The dependence of $k_{\text{off}}$ on Mg$^{2+}$ was also determined

### Table II

| Data set | GTP$\gamma$S$\cdot$Mg$^{2+}$ | $\alpha$/$\gamma$ |
|----------|-----------------------------|-------------------|
| Resolution (Å) | 18–2.1 (2.2–2.1) | 18–2.6 (2.7–2.6) |
| $R_{\text{free}}$ | 0.25 (0.30) | 0.28 (0.40) |
| $R$-factor | 0.19 (0.27) | 0.24 (0.36) |

Root-mean-square deviation in:
- Bond angles (%) 1.566 1.662
- Bond distances (Å) 0.013 0.013
- Ramachandran plot analysis:
  - Most favored (%) 94.2 86.3
  - Allowed (%) 9.4 12.8
  - Disallowed (%) 0.0 0.0

### Table III

| Ligand | G-protein complex | Trapping ligand | $k_{\text{cat,GTP}}$ | $k_{\text{cat,GDP}}$ |
|--------|-------------------|----------------|---------------------|---------------------|
| GDP-AMF | G$_{i\alpha 1}$-GDP-AMF | GTP$\gamma$S | 2.0 ± 0.3 | 0.05 ± 0.01 |
| GDP | G$_{i\alpha 1}$-GDP | GTP$\gamma$S | 2.1 ± 0.3 | ND |
| GDP | G$_{i\alpha 1}$-GDP | GTP$\gamma$S | 0.087 ± 0.008 | ND |
| GDP | G$_{i\alpha 1}$-GDP | GTP$\gamma$S | 3.5 ± 0.7 | ND |
| GDP | G$_{i\alpha 1}$-GDP | GTP$\gamma$S | 0.014 ± 0.003 | ND |
| GDP | G$_{i\alpha 1}$-GDP | GTP$\gamma$S | 4.2 ± 0.3 | 0.12 ± 0.02 |
| GDP | G$_{i\alpha 1}$-GDP | GTP$\gamma$S | Very slow | Very slow |

* Indicates the ligand bearing the label. See “Materials and Methods” for details.

For A326S G$_{i\alpha 1}$ ($\bullet$, alone; $\square$, + $\beta\gamma$), protein was first incubated with radiolabeled GDP for 30 min at 30 °C prior to addition of unlabeled GTP-$\gamma$S. Data are presented as percentage of the maximal amount of [35S]GTP-$\gamma$S bound. For wild-type G$_{i\alpha 1}$ ($\bullet$, alone; $\square$, + $\beta\gamma$), [35S]GTP-$\gamma$S was used as the competing ligand, but data are reported as the percentage of the maximum amount of GDP bound. Maximum nucleotide binding corresponded to 60% and 10% of the total protein present (200 nM) for the wild-type and mutant proteins, respectively.

FIG. 2. Dissociation of GDP from wild-type and A326S G$_{i\alpha 1}$. For A326S G$_{i\alpha 1}$ ($\bullet$, alone; $\square$, + $\beta\gamma$), protein was first incubated with radiolabeled GDP for 30 min at 30 °C prior to addition of unlabeled GTP-$\gamma$S. Data are presented as percentage of the maximal amount of [35S]GTP-$\gamma$S bound. For wild-type G$_{i\alpha 1}$ ($\bullet$, alone; $\square$, + $\beta\gamma$), [35S]GTP-$\gamma$S was used as the competing ligand, but data are reported as the percentage of the maximum amount of GDP bound. Maximum nucleotide binding corresponded to 60% and 10% of the total protein present (200 nM) for the wild-type and mutant proteins, respectively.

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![Image](https://via.placeholder.com/150)
and co-workers (4) noted that the A366S mutation in Gs
reasoned that the loss of Gs type and mutant proteins are inactivated at 30 °C with rates of
linear fits of the last five data points of each condition.

A326S Gi

C

A

B

C

B

A

GTPase assays were conducted at 30 °C for wild-type Gi—( ), and A326S Gi—( ) as described under “Materials and Methods.”

Irreversible Inactivation of A326S Gi—Iiri and co-workers (4) noted that the A366S mutation in Gi accelerates irreversible inactivation (apparent denaturation) of the protein such that it is unable to bind GTPγS. The authors reasoned that the loss of Gi in non-testicular tissues was due to accelerated inactivation of the unliganded (nucleotide-free) α subunit at 37 °C. We have conducted similar experiments with wild-type and A326S Gi. In the absence of added nucleotide or the βγ subunit heterodimer, both the wild-type and mutant proteins are inactivated at 30 °C with rates of 0.012 ± 0.003 and 0.047 ± 0.004 min⁻¹, respectively (Fig. 6A).

At 37 °C, these rates are increased roughly 8- and 5-fold (Fig. 6B). High concentrations of GDP slow inactivation but do not prevent it, even at millimolar concentrations (Fig. 6C). Notably, both proteins are quite stable at 30 °C in the presence of βγ (Fig. 6A). However, the mutant protein is inactivated rapidly at 37 °C in the presence of βγ (k = 0.061 ± 0.005 min⁻¹), even though the wild-type heterotrimer is still reasonably stable at this temperature (k = 0.005 ± 0.001 min⁻¹) (Fig. 6C). Both wild-type and mutant proteins are stable when activated with GTPγS or AlF₄⁻ (data not shown).

Structural Characterization of A326S Gi—We crystallized and determined structures of the mutant Gi subunit in two different complexes. A least squares superposition of the Cα atoms of each complex on those of the corresponding wild-type structure results in root mean square deviations of 0.20 Å for the A326S Gi-GTPγS-Mg²⁺ complex and 0.16 Å for the mutant heterotrimer. Thus, there are no global changes in the architecture of the mutant subunit. At a resolution of 2.1 Å, the A326S Gi-GTPγS-Mg²⁺ complex provides the greatest insight into how the mutation is accommodated and nucleotide binding is destabilized.

The active site of the mutant structure contains a single molecule of GTPγS and one magnesium ion. During initial refinement, the magnesium ion and the nucleotide were excluded from the model. Subsequent inspection of the SigmaA weighted Fo - Fc map (18) clearly showed the location of the nucleotide in the binding pocket, which is made up of residues from the Ras-like and the α-helical domains (Fig. 7, A and B). In difference maps contoured at the 3 σ level (Fig. 7A), electron density around the base is broken in the region adjacent to the side chain of Ser-326, and for the ribose ring, only the O2' and O3' oxygen atoms show strong density. Only the α and β phosphates appear as strong peaks in the map of residuals. The density is quite broken even at the position of the γ phosphate, and peaks are confined to the oxygen atoms (Fig. 7A). In the structure of the wild-type a subunit, the nucleotide is one of the best defined regions with a mean B-factor of ~9.0 Å², significantly less than the average B-factor (~17 Å²) for the model. However, B-factors of the nucleotide in the mutant a subunit structure (~21 Å²) approach the mean B-factor of the model (24 Å²). Density for the magnesium ion was also weak in both 2Fo - Fc and Fo - Fc maps. Although the Mg²⁺ site appeared to be poorly occupied, inclusion of the metal in the model at full occupancy and subsequent refinement did not produce significant negative difference density at the binding site. After B-factor and occupancy refinement, the magnesium ion has an occupancy of 0.83 and a relatively high B-factor (30 Å²), which is in accord with the poor definition of the site in 2Fo - Fc maps.

Ala-326 is situated in a loop spanning β6 and α5 in the Ras-like domain of the α subunit (Fig. 7C). This loop normally

for A326S Gi (Fig. 5). The apparent Kᵦ for Mg²⁺ is 25 ± 7 nM, in good agreement with values obtained previously for the wild-type protein (21).

\[ K_\gamma = 25 \pm 7 \text{ nM} \]

Irreversible Inactivation of A326S and Wild-type Gᵦ—The final concentrations of protein and nucleotide were 50 nm and 40 μM, respectively.
A326S \( G_{\alpha_1} \) Approximates Receptor-bound State

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**FIG. 6. Irreversible inactivation of wild-type and A326S \( G_{\alpha_1} \).** Experiments with GDP-bound wild-type \( G_{\alpha_1} \) ( ), or A326S \( G_{\alpha_1} \) ( ), were conducted in the absence of added GDP at 30 °C (A) or 37 °C (B), and the nucleotide dependence of inactivation was examined for the mutant at 30 °C (C). Aliquots (30 μl) of \( G_{\alpha_1} \) (400 nM) were removed at the indicated times, combined with an equal volume of assay buffer provides hydrogen-bonding interactions from the backbone nitrogen atom of residue 326 to the O6 atom of the purine base (Fig. 7B). In the A326S mutant, the side chain of the serine residue is oriented away from the nucleotide such that its Oγ hydroxyl is directed into a hydrophobic pocket in the core of the Ras-like domain, where it hydrogen bonds with Asn-269. In addition, the serine hydroxyl forms van der Waals contacts with an aliphatic carbon (Cγ1) in the side chain of Ile-49, the carbonyl oxygen of Gly-45, and the N7 atom of the guanine ring. Despite these contacts, there is no significant displacement of any residues around the serine side chain. Instead, the van der Waals contacts formed by the serine hydroxyl force the main chain and Cβ carbon of Ser-326 into closer contact with the purine ring. The number of van der Waals contacts between the backbone atoms of residue 326 and the purine ring is twice that observed for Ala-326 in the wild-type complex.

Although the pattern of hydrogen bonds in the mutant is altered compared with the wild-type structure, there is no net change in the number of hydrogen bonds between the protein and nucleotide (Fig. 7B). For example, van der Waals contacts between the Ser-326 hydroxyl group and the main chain carbonyl of Gly-45 lead to a loss of a hydrogen bond between the backbone nitrogen of the latter residue and the β phosphate oxygen (O1B). However, Arg-178 has also changed conformation, such that it is positioned beside the β phosphate opposite Gly-45. In this position, the side chain makes a new hydrogen bond with the bridging oxygen between the β and γ phosphates and thereby offsets the lost interaction with Gly-45.

The structure of the A326S \( G_{\alpha_1} \)-GDP-βγ complex was determined at lower resolution (2.6 Å) than that of the corresponding wild-type heterotrimer. At this level of resolution, the structure of the heterotrimeric complex formed by A326S \( G_{\alpha_1} \) is indistinguishable from that containing the wild-type α subunit, save for the occupancy of the nucleotide site (see “Materials and Methods”). Refinement tests indicate that only approximately one-third of the heterotrimers are bound to GDP in the crystal. Less consistent with the refinement statistics is a model in which GDP is present at all binding sites but is highly disordered (B > 100 Å²). Intermediate models are plausible; the data do not permit an accurate estimate of nucleotide occupancy or thermal factor, which are highly correlated at moderate resolution. Otherwise, all of the GDP-protein contacts observed in the wild-type heterotrimer are evident in the mutant, indicating that the nucleotide binding site is undistorted by the A326S mutation in the complex with GDP and βγ.

**DISCUSSION**

Several mutations have been shown to reduce the affinity of G protein α subunits for guanine nucleotides, but only a few destabilize GDP binding selectively, with little or no effect on binding of GTP-S-Mg²⁺ (4, 22). Given the location of these mutations near the carboxyl termini of \( G_{\alpha} \) proteins, it has been suggested that these proteins, C325S \( G_{\alpha} \) and A326S \( G_{\alpha} \), mimic the effect of agonist-activated receptors on guanine nucleotide exchange by the α subunits. This hypothesis seems reasonable, since receptors selectively destabilize GDP binding containing [35S]GTP-S, and incubated for 30 min at 30 °C prior to determination of the amount of bound radioactive nucleotide. Inactivation of wild-type and mutant \( G_{\alpha_1} \) was monitored in the presence (filled symbols) or absence (open symbols) of 400 nM βγ (A and B). In C, GDP was included in the assay at 5 μM ( ), 10 μM ( ), or 1 mM ( ). In the experiment with 1 mM GDP, free GDP was removed by rapid gel filtration prior to nucleotide exchange. A control (○, GDP omitted) was included to demonstrate that this additional step did not change the observed rate of inactivation of the α subunit.
and the carboxyl termini of Gα proteins are important sites for receptor interaction; however, this domain appears to play no role in GTP hydrolysis or effector binding (1–3).

Excluding interactions with the receptor, we have determined the dynamics of the GTPase cycle for A326S Gα1. Several aspects of the cycle are unaffected by the mutation. The binding of GTPγS-Mg2⁺ to the mutant α subunit is essentially irreversible, its apparent affinity for Mg2⁺ is normal, its kcat for GTP hydrolysis is indistinguishable from the characteristic wild-type value, and its interactions with adenyl cyclase are appropriate (data not shown). However, the rate of dissociation of GTPγS from A326S Gα1 in the absence of Mg2⁺ is accelerated substantially compared with the wild-type protein. Further, accelerated guanine nucleotide exchange by the mutant α subunit permits accumulation of a substantial amount of GTP-Mg2⁺-α under steady-state conditions (40% of total α). In the absence of a GTPase-activating protein, this complex should interact productively with effectors in vivo, and this can account for the testotoxicosis observed in patients harboring the corresponding mutation in Gα1. Of interest, GTPase-activating proteins have been identified for members of the Gα and Gα subfamilies of Gα proteins, but not for Gα1 (23).

The most striking perturbations introduced by the mutation are manifest in the kinetic properties of the heterotrimer. Previous studies of the wild-type heterotrimer have defined a positive synergism between the βγ heterodimer and GDP. Although they do not interact directly with each other, GDP and βγ influence regions of the α subunit (the so-called switch regions) that are important for association of α with βγ and nucleotide and for protein stability. In the GDP-bound state, the switch I and II regions of Gα1 are flexible and adopt requisite conformation for association with βγ. However, activating ligands (GTPγS-Mg2⁺ or GDP-AMF) force these same peptide segments into rigid conformations that do not favor complexation with βγ. In a complementary fashion, βγ enhances the affinity of Gα1 for GDP by allosteric enforcement of the interactions between protein and nucleotide (9). In addition, our data and those of others (24) demonstrate that interactions between Gα and βγ reduce susceptibility of the Gα-GDP complex to irreversible inactivation.

A large part of this positive synergism is lost in the mutant heterotrimer. At 30 °C, the rate of dissociation of GDP from the heterotrimer is increased roughly 250-fold. Nevertheless, βγ still stabilizes Gα against irreversible inactivation. Uncoupling of these roles of βγ suggests that independent structural mechanisms of action may be at work in the wild-type protein. Gross conformational changes in the mutant heterotrimer were not detected in the structures presented here. In particular, the mutant heterotrimer displays the normal, βγ-induced formation of an ion pair between Arg-178 and Glu-43 of Gα1, that encapsulates GDP in the heterotrimer (9). Loss of this structural feature could have accounted for the loss of positive cooperativity in the binding of βγ and GDP to A326S Gα1. Thus, subtle alterations not detectable at the resolution to which the structure was determined may lead to separation of βγ-mediated functions in the heterotrimer.

The rate of irreversible inactivation of Gα1 is enhanced by the mutation. Moreover, the capacity of βγ to prevent irreversible inactivation is diminished at 37 °C. The reduced stability of the mutant heterotrimer at this temperature suggests that this is most likely the form of the G protein that accounts for the temperature-sensitive phenotype of the mutation in Gα1. Based on this observation and nucleotide-binding data, one might conclude that irreversible inactivation represents a decay of a rapid equilibrium between bound and free states of Gα that results from facile, irreversible denaturation of the nucleo-
otid-free form. However, high concentrations of GDP (millimolar) do not prevent inactivation, as predicted by this simple model. Although we have analyzed the data as a first-order process, the mechanism must be more complex.

From a structural standpoint, stabilization of $\alpha$ by activators or $\beta\gamma$ occurs in part through a common mechanism: structural organization of switch I and II. Although conformational flexibility in these regions is requisite for the transition between active and inactive states of $G_{\alpha}$, it also appears to doom the GDP-bound protein to a folded or denatured form that is permanently incapacitated. Unlike activated forms of $G_{\alpha}$, the mutant and wild-type heterotrimers both exhibit irreversible inactivation at 37 °C.

The structure of the A326S $G_{i\alpha1}$-GTPγS complex provides an attractive, albeit subtle model for receptor-mediated nucleotide exchange. Although the serine hydroxyl group in the mutant makes van der Waals contact with N7 of the guanine ring, it does not displace the nucleotide as suggested (4). Rather, it is inserted into a hydrophobic pocket where it hydrophobically stabilizes the nucleotide-free conformation of the heterotrimer, rather than inducibly destabilizing nucleotide binding from the “backside” of the binding pocket. If this is truly the case, then the mutation approximates this role of the receptor. The degree of destabilization achieved ($k_{\text{off, GDP}} \sim 3/\text{min}$) is also comparable to that caused by receptor agonists when $G$ proteins are reconstituted in phospholipid vesicles with receptors ($k_{\text{off, GDP}} \sim 9/\text{min}$) (25). In crystals, the nucleotide binding site is undistorted in the mutant heterotrimer: the state in which the $\alpha$ subunit is presented to the receptor. Lattice forces may reinforce the structure of the nucleotide-free binding site in the absence of GDP. However, it is also possible that the A326S mutation stabilizes the nucleotide-free conformation of the heterotrimer, rather than inducibly destabilizing nucleotide exchange in the GDP-bound form, since little GDP is retained in these crystals. In either case, it appears that A326S $G_{i\alpha1}$ Approximates Receptor-bound State

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REFERENCES

1. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
2. Conklin, B. R., and Bourne, H. R. (1993) Cell 73, 631–641
3. Sprang, S. R. (1997) Annu. Rev. Biochem. 66, 629–678
4. Iiri, T., Herzmark, P., Nakamoto, J. M., Van Dop, C., and Bourne, H. R. (1994) Nature 371, 164–168
5. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
6. Johnson, R. A., and Walsht, T. F. (1979) Adv. Cyclic Nucleotide Res. 10, 135–167
7. Lee, E., Linder, M. E., and Gilman, A. G. (1994) Methods Enzymol. 237, 146–164
8. Linder, M. E., and Mumby, S. M. (1994) Methods Enzymol. 237, 254–268
9. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) Cell 83, 1047–1058
10. Northup, J. K., Smigel, M. D., and Gilman, A. G. (1982) J. Biol. Chem. 257, 11416–11423
11. Brandt, D. R., Asano, T., Pedersen, S. E., and Ross, E. M. (1983) Biochemistry 22, 4357–4362
12. Higashijima, T., Ferguson, K. M., Smigel, M. D., and Gilman, A. G. (1987) J. Biol. Chem. 262, 757–761
13. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) Science 265, 1405–1412
14. Otwinowski, Z., and Minor, W. (1996) Methods Enzymol. 276, 307–326
15. Navaza, J. (1994) Acta Crystallogr. Sect. A 50, 157–163
16. Brunger, A. T. (1992) X-PLOR Version 3.1: A System for X-ray Crystallography and NMR, Yale University Press, New Haven, CT
17. Brunger, A. T. (1990) Acta Crystallogr. Sect. A 46, 46–57
18. Read, R. J. (1986) Acta Crystallogr. Sect. A 42, 140–149
19. Jones, T. A., and Kjeldgard, M. (1996) O, version 5.1, Uppsala University, Uppsala, Sweden
20. Higashijima, T., Graziano, M. P., Suga, H., Kainosho, M., and Gilman, A. G. (1991) J. Biol. Chem. 266, 3386–3401
21. Higashijima, T., Ferguson, K. M., Sternweis, P. C., Smigel, M. D., and Gilman, A. G. (1987) J. Biol. Chem. 262, 762–766
22. Thomas, T. C., Schmidt, C. J., and Neer, E. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10295–10299
23. Berman, D. M., and Gilman, A. G. (1998) J. Biol. Chem. 273, 1269–1272
24. Katada, T., Onuma, M., and Ui, M. (1986) J. Biol. Chem. 261, 8182–8191
25. Asano, T., and Ross, E. M. (1984) Biochemistry 23, 5467–5471