New Insights into the Role of Conserved, Essential Residues in the GTP Binding/GTP Hydrolytic Cycle of Large G Proteins*

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The GTP hydrolytic (GTPase) reaction terminates signaling by both large (heterotrimeric) and small (Ras-related) GTP-binding proteins (G proteins). Two residues that are necessary for GTPase activity are an arginine (often called the “arginine finger”) found either in the Switch I domains of the α subunits of large G proteins or contributed by the GTP-activating proteins of small G proteins, and a glutamine that is highly conserved in the Switch II domains of Gα subunits and small G proteins. However, questions still exist regarding the mechanism of the GTPase reaction and the exact role played by the Switch II glutamine. Here, we have characterized the GTP binding and GTPase activities of mutants in which the essential arginine or glutamine residue has been changed within the background of a Gα chimera (designated αC+), comprising mainly of the α subunit of retinal transducin (αT) and the Switch III region from the α subunit of Gs. As expected, both the αC+/(R174C) and αC+/(Q200L) mutants exhibited severely compromised GTPase activity. Neither mutant was capable of responding to aluminum fluoride when monitoring changes in the fluorescence of Trp-207 in Switch II, although both stimulated effector activity in the absence of rhodopsin and Gβγ. Surprisingly, each mutant also showed some capability for being activated by rhodopsin and Gβγ to undergo GDP→GTP exchange. The ability of the mutants to couple to rhodopsin was not consistent with the assumption that they contained only bound GTP, prompting us to examine their nucleotide-bound states following their expression and purification from Escherichia coli. Indeed, both mutants contained bound GDP as well as GTP, with 35–45% of each mutant being isolated as GDP-Pi complexes. Overall, these findings suggest that the R174C and Q200L mutations reveal Gα subunit states that occur subsequent to GTP hydrolysis but are still capable of fully stimulating effector activity.

The visual phototransduction cascade in retinal rod outer segments consists of the heterotrimeric G protein, transducin, its upstream receptor rhodopsin, and its downstream effector, the cGMP-phosphodiesterase (PDE). This is a highly sensitive signaling system, capable of detecting a single photon of light, which excites the seven-membrane spanning receptor, rhodopsin. Light-activated rhodopsin binds and activates the α subunit of transducin (αT), enabling it to bind GTP. Activated GTP-bound αT then stimulates PDE activity by altering the positions of its regulatory γ subunits (γY355E), thereby allowing its catalytic subunits (αPDE and βPDE) to hydrolyze cGMP. The reduction in the cellular levels of this second messenger causes cation-specific cGMP-gated ion channels in the outer segments to close, leading to the hyperpolarization of rod outer segment membranes and the generation of the visual response. There is an amplification of the signal at every step of this cascade such that a single photon of light can lead to the inhibition of 10⁷-10⁸ Na⁺ ions from entering the rod cell (1).

Members of the family of large G proteins are made up of three distinct subunits: α (39–46 kDa), β (36 kDa), and γ (8 kDa), with β and γ remaining tightly associated under all non-denaturing conditions (often referred to as the Gβγ complex). The N-terminal region of Gα subunits, together with the C-terminal region of Gγ subunits, have lipid modifications and are involved in membrane attachment (1–3). G proteins are inactive and unable to propagate signals when their α subunits are in the GDP-bound state. Receptor-catalyzed exchange of GDP for GTP on the Gα subunit can lead to the dissociation of the Gβγ complex from the GTP-bound Gα subunit, allowing both Gα and Gβγ to engage downstream effectors. The GTPase reaction terminates the signaling functions of large as well as small G proteins by acting as an internal clock, which controls the activated lifetime of the G protein and its associated physiological response.

Among the more fundamentally important but still unanswered questions regarding G protein function concerns the steps that underlie the GTPase reaction and signal termination. Two residues have been consistently implicated as being important in the GTPase reaction (4–6). One is a conserved arginine residue, often referred to as the “arginine finger,” that is found either in the helical domain/Switch I region of the Gα subunits of large G proteins or contributed in trans by GTPase-activating proteins for small G proteins (5). This arginine residue is ADP-ribosylated by cholera toxin in the Gαs subunit, resulting in an inhibition of GTPase activity and constitutive activation of adenyl cyclase (7). The other frequently implicated residue is the highly conserved glutamine found in the Switch II regions of both large and small G proteins. Mutations of this glutamine residue in Ras (Gln-61) have been detected in human tumors and are carried by some animal retroviruses (7, 8). Moreover, a variety of human tumors have been associated with mutations of either the Switch II glutamine or the arginine finger in the Gαi and Gαs proteins (7, 9, 10).

Although there seems little doubt that these two residues have important roles in the GTPase reactions of both large and small G proteins, there are questions regarding exactly how they participate in the catalytic mechanism and whether they serve the same function in all G proteins. Much of what has been proposed regarding the functions of these residues comes from the x-ray crystal structures of Gα subunits bound to AlF₄⁻, as well as from the corresponding structures for ternary complexes formed between small G proteins, AlF₄⁻, and GTPase-activating proteins, as these have been proposed to mimic the transition state for the GTPase reaction (4–6,11). Based on these structures, it has...
been suggested that the positively charged arginine residue stabilizes negative charges that develop on GTP during the transition state for the GTPase reaction (12, 13), whereas the Switch II glutamine residue was originally suggested to be responsible for correctly positioning the water molecule for nucleophilic attack (13) and to act as a general base catalyst for the hydrolytic reaction. However, more recent studies have called some of these suggestions into question. For example, mutations of the conserved Switch II glutamine residue in Ras (Gln-61) did not alter the position of the bound water (14), and when substituted with an unnatural amino acid (i.e. a nitro-analog of glutamine), which is a much weaker base compared with glutamine itself, there was little effect on GTP hydrolysis (15). Moreover, computer simulations have demonstrated that Gln-61 is unlikely to serve as the general base (16), and recently it has been argued that GTP hydrolysis occurs by substrate-assisted catalysis, with the γ-phosphate of GTP serving as the general base catalyst (17).

Thus, the original motivation for these studies was to further examine the roles played by the conserved arginine and glutamine residues in the GTP-binding/GTPase cycles of large G proteins. Mutations of Arg-174 to cysteine and Gln-200 to leucine were made in the background of an α2/α1 chimera that contained wild-type α2 residues except in the Switch III domain where residues 215–294 were from α1 (see “Experimental Procedures”). This chimera, designated from here on as α2*, was used because, unlike wild-type α2, it can be readily expressed and purified from Escherichia coli and retains the ability to undergo rhodopsin- and Gβγ-promoted GDP-GTP exchange and to activate the cGMP PDE. During the course of these studies, we obtained some interesting new insights regarding the individual steps comprising the activation (GDP-GTP exchange)-deactivation (GTPase) cycles of large G proteins. As expected, we found that both the R174C and Q200L mutants exhibited severely compromised rates of GTP hydrolysis and were unable to assume an AlF4−-induced transition state conformation. Consequently, these α2* mutants were capable of constitutively stimulating PDE activity (i.e. without the addition of light-activated rhodopsin, Gβγ, and GTP). All of these findings were consistent with our initial assumption that both the R174C and Q200L mutants were isolated in the GTP-bound state. However, surprisingly, we found that a fraction of each mutant was capable of responding to rhodopsin and Gβγ to undergo GDP-GTP exchange. This seemed inconsistent with the idea that the R174C and Q200L mutants, upon isolation, existed entirely in the GTP-bound state. Indeed, we found that a significant percentage of each mutant contained bound GDP with a sizeable fraction also containing inorganic phosphate (Pγ) and thus existed as GDP-Pγ complexes. Given that each mutant was able to fully activate PDE with a dose-response profile essentially indistinguishable from wild-type α2*, all three forms of the R174C and Q200L mutants (GTP-bound, GDP-bound, and GDP-Pγ complexes) must represent signaling-competent conformations. Thus, these findings highlight the existence of conformational states that occur subsequent to the GTP hydrolytic reaction (representing GDP-Pγ and GDP-bound forms of Gα subunits) and are still capable of regulating target/effectors. They also demonstrate for the first time that the conserved arginine and glutamine residues are not only necessary for the actual GTP hydrolytic step but also contribute to product (Pγ) release and influence the rate at which these different conformational states are converted back to the signaling-inactive GDP-bound state.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Sense and antisense oligonucleotide primers were synthesized with the desired mutated base in the center of the primer. The QuikChange site-directed mutagenesis kit (Stratagene) was used to carry out the PCR-based mutagenesis on miniprep plasmid DNA. The parental DNA template was digested using DpnI endonuclease, and the nicked vector DNA with the desired mutation was transformed into Epicurian Coli XL-1 Blue supercompetent cells. DNA was purified from single colonies using the Qiagen plasmid miniprep kit. The DNA was sequenced at the Bio Resource Center, Cornell University.

**Construction of the α2* Chimera**—The bovine retinal α2 subunit, when expressed in E. coli, is not soluble, whereas the Gαi3 subunit is soluble and fully functional. There are several αi1 and αi2 chimeras available, which can be expressed in E. coli and bind GTP similar to retinal αi but are unable to stimulate the cyclic GMP PDE (18). We have used a construct designated pHis6Chi6, which was obtained from Dr. Heidi Hamm (Vanderbilt University). This chimera, which has the corresponding region from αi1 inserted between residues 215 and 294 of αi2, is unable to stimulate PDE activity but is capable of binding to αi2. By changing residues 244 and 247 back to the original amino acids in αi2, the ability to stimulate PDE activity was restored to at least 50% of the levels obtained with retinal αi. This form of transducin has been designated αi2*. The R174C and Q200L mutants have been made in the αi2* background.

**Protein Expression and Purification**—The recombinant wild-type αi2* subunit and the αi2* mutants were expressed in BL21 (DE3) supercompetent cells and purified as described (18, 19). The proteins were further purified by gel filtration chromatography on a HiLoad Superdex 75 HR26/60 column equilibrated with a buffer containing 20 mM Na-Hepes, pH 7.5, 5 mM MgCl2, and 1 mM Na3VO4. The samples were aliquotted, snap-frozen, and stored at −80 °C. The final yield of chimeric αi2* was typically ~5 mg of pure protein/liter of bacterial culture.

**Purification of Retinal Proteins**—Frozen, dark-adapted bovine retina were obtained from W. L. Lawson Co. (Lincoln, NE). Holo-transducin and PDE were purified from rod outer segment membranes as outlined in (20). The PDE was further purified by gel filtration chromatography on a HiLoad Superdex 200 HR26/60 column equilibrated with a buffer containing 20 mM Na-Hepes, pH 7.5, and 10% glycerol. Holo-transducin was loaded onto a Blue-Sepharose column (21) to separate the Gβγ subunit complex from α2*. This form of transducin has been applied to a 50-ml Blue-Sepharose column that had been pre-equilibrated with 1 G1G0A buffer (1 G1G0A buffer + 100 mM KCl) to elute the Gβγ complex. The bound α2* was eluted from the column with 250 ml of high salt buffer (G1G0A buffer + 500 mM KCl). The α2* and Gβγ subunits were further purified by a final round of gel filtration chromatography on a HiLoad Superdex 75 HR26/60 column. The samples were aliquotted, snap-frozen, and stored at −80 °C. The final yield of each subunit ranged from 3 to 5 mg of pure protein/300 retinas. Urea-washed rod outer segment membranes highly enriched in rhodopsin were prepared as previously described (22), and the rhodopsin was solubilized in 0.01% dodecyl maltoside (from here on referred to simply as rhodopsin).

**[^35S]GTPγS Binding Assays**—Light-activated rhodopsin (R*), together with αi2* and the Gβγ complex, was incubated in HMDM buffer (20 mM Hepes, pH 7.4, 5 mM MgCl2, 0.01% dodecyl maltoside) for 20 min at room temperature and in room light. [^35S]GTPγS (final concentration = 1 μM; specific activity = 1 Ci/mmol) was added to initiate the reaction. The reaction was quenched by direct application to prewetted nitrocellulose filters (Schleicher & Schuell, pore size = 0.45 μm) on a suction manifold. The filters were washed twice with HM buffer (20 mM Hepes, pH 7.5, 5 mM...
MgCl₂), added to scintillation liquid (30% LSC Scintisafe Mixture), and counted in a scintillation counter (LS6500 multipurpose scintillation counter).

Measurements of cGMP PDE Activity — The analysis of cGMP hydrolysis by the retinal PDE was carried out as outlined in Ref. 23. A pH microelectrode was used to measure the decrease in pH (in millivolts) that results from the release of one proton for each molecule of cGMP hydrolyzed by PDE. In a typical assay, α₄₄₅ was preincubated with Gβγ, R*, GTPγS and PDE in a buffer containing 5 mM Hepes, pH 7.4, 2 mM MgCl₂, 100 mM NaCl, and 0.01% dodecyl maltoside, at room temperature and in room light for 60 min. The reaction was initiated by the addition of the substrate, cGMP, to a final concentration of 5 mM, and the change in pH was monitored over time. At the end of the assay period (100 s), the buffering capacity (mV/nmol) was determined by the addition of 0.1 mol of NaOH to the reaction mixture. The rate of hydrolysis of cGMP (nmol/s) was determined from the ratio of the slope of the pH record (mV/s) and the buffering capacity of the assay buffer (mV/nmol).

GTPase Assays — Light-activated rhodopsin, α₄₄₅, and Gβγ were incubated in HMDM buffer for 20 min at room temperature and in room light. The assays were initiated by the addition of [γ³²P]GTP (final concentration = 1 μM; specific activity = 13 Ci/mmol). Aliquots (40 μl) were removed at specific time points and added to 1 ml of 5% activated charcoal (neutralized) in 50 mM NaH₂PO₄. The samples were centrifuged at high speed, and then an aliquot of the supernatant (100 μl) was added to scintillation fluid and counted.

Fluorescence Measurements — The binding of aluminum fluoride (AlF₄⁻) as well as GTPγS was monitored by measuring the intrinsic tryptophan fluorescence of α₄₄₅ upon excitation at 300 nm and emission at 345 nm (24, 25). In the experiments examining the effects of AlF₄⁻, 300 nM α₄₄₅ was preincubated in HM buffer at room temperature. The tryptophan fluorescence emission of α₄₄₅ was monitored for 1 min, at which time AlF₄⁻ (prepared by mixing 250 mM NaF and 150 μM AlCl₃) was added. A similar procedure was used for the GTPγS-binding experiments, where 300 nM α₄₄₅ was preincubated with Gβγ and R* in HMDM buffer at room temperature, for 10 min. The fluorescence measurements were carried out using a Varian eclipse spectrofluorometer.

Trypsin Proteolysis — The patterns of tryptic proteolysis were determined as previously described (26). The α₄₄₅ subunit (10 μg) was treated with/without AlF₄⁻ for 30 min at room temperature. The protein was then mixed with 0.1 μg of l-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin and incubated at room temperature for 20 min. Digestion was terminated by the addition of 0.2 μg of soybean trypsin inhibitor, followed by SDS sample buffer and then boiled for 5 min. The proteolytic fragments were resolved by SDS-polyacrylamide gel (15%) electrophoresis (27) and stained with Coomassie Blue.

HPLC Analysis — The nucleotide analyses were performed using a Sunfire C-18 reversed phase column (0.46 × 25 cm) filled with 5 μm (particle size) silica (Waters). The system consisted of a Waters 1525 Binary HPLC pump machine and a Waters 2487 dual absorbance UV detector. The column was equilibrated with 100 mM KH₂PO₄, pH 6.5, 10 mM tetrabutylammonium bromide, 0.2% NaN₃, and 7.5% acetonitrile, and isotropic elution was performed at ambient temperature with a flow rate of 1 ml/min. In this system, the order of elution of guanine nucleotides was GMP, GDP, and GTP (retention times 4.8, 8, and 14 min, respectively). In a typical experiment, 100 μM α₄₅ was boiled for 3 min at 95 °C and centrifuged at high speed for 10 min to remove denatured protein. An aliquot of the supernatant (20 μl) was injected into the column. The column was calibrated with 100 μM solutions of the different guanine nucleotides.
It has been proposed that aluminum fluoride induces conformational changes within G proteins that mimic their transition state for the GTPase reaction (4–6). These conformational changes occur within seconds and, in the case of \( \alpha^T \), can be monitored in real time by an increase in Trp-207 fluorescence. An example is shown in Fig. 2. Both the \( \alpha^T \)(Q200L) mutant and the \( \alpha^T \)(R174C) mutant were incapable of undergoing an AlF\(_4^-\)-induced conformational change. These findings are in line with the x-ray crystal structures for Ga-GDP-AlF\(_4^-\) complexes showing that both the Gln-200 and Arg-174 residues contact AlF\(_4^-\) (4), and so substitutions at these sites would be expected to severely compromise the stabilization of the transition state complex for the GTPase reaction.

The \( \alpha^T \)(R174C) and \( \alpha^T \)(Q200L) Mutants Are Capable of Stimulating the Effector Enzyme, the Cyclic GMP PDE in the Absence of Rhodopsin, G\( \delta \)\( \gamma \), and GTP\( \gamma \)S—Fig. 3 shows the results of experiments comparing the abilities of the \( \alpha^T \) subunit and the \( \alpha^T \)(R174C) and \( \alpha^T \)(Q200L) mutants to stimulate cyclic GMP hydrolysis by the PDE. Whereas wild-type \( \alpha^T \) was only able to stimulate PDE activity in the presence of R* and G\( \delta \)\( \gamma \), and upon the addition of GTP\( \gamma \)S, both the R174C and Q200L mutants appeared to be capable of maximally stimulating the PDE, even in the absence of R*, G\( \delta \)\( \gamma \), and GTP\( \gamma \)S (Fig. 3A). Fig. 3B shows the dose-response profiles for the different \( \alpha^T \) subunits. Each mutant, in the absence of R* and G\( \delta \)\( \gamma \) (as well as GTP\( \gamma \)S), was as potent as rhodopsin- and G\( \delta \)\( \gamma \)-activated wild-type \( \alpha^T \) in stimulating the PDE. These results indicate that changing either Arg-174 or Gln-200 did not compromise the binding and regulation of the target/effector by \( \alpha^T \) and that the mutants were constitutively active in stimulating the effector enzyme.

Trypsin-proteolysis Profiles for the \( \alpha^T \)(R174C) and \( \alpha^T \)(Q200L) Mutants Differ from Those for Activated \( \alpha^T \)—The susceptibility of Ga subunits to trypsin proteolysis offers a sensitive assay for monitoring conformational changes in Switch II that have been correlated with activation (26). In the case of either native retinal \( \alpha^T \) or the recombinant wild-type \( \alpha^T \) subunit, the binding of GTP or AlF\(_4^-\) causes Arg-204 in Switch II to move from a solvent-exposed environment to a less accessible position, thereby protecting it against proteolysis. Exposure of GDP-bound \( \alpha^T \) (or \( \alpha^T \)) to trypsin yields an ~23-kDa fragment, whereas the identical treatment of GTP- or AlF\(_4^-\)-bound \( \alpha^T \) (or \( \alpha^T \)) gives rise to an ~32-kDa fragment.

Fig. 4 shows that although wild-type \( \alpha^T \) exhibited the characteristic trypsin-proteolysis profile, it was surprising that the \( \alpha^T \)(R174C) and \( \alpha^T \)(Q200L) mutants were only partially protected from proteolysis, so that in both cases, the 23- and 32-kDa fragments were detected. The trypsin sensitivity of the mutants was unaffected by the addition of AlF\(_4^-\), consistent with their inability to respond to this reagent (Fig. 2). However, the fact that these mutations resulted in only partial rather than full protection against trypsin treatment was initially puzzling, because we assumed that the R174C and Q200L mutants were predomin-
GTPase Reaction of Transducin

FIGURE 5. Trypsin proteolysis profiles for wild-type $\alpha_T^*$, $\alpha_T^*$(R174C), and $\alpha_T^*$(Q200L). The wild-type $\alpha_T^*$ subunit, $\alpha_T^*$(R174C), or $\alpha_T^*$(Q200L) (10 $\mu$g of each) was treated with or without AlF$_4^-$ for 30 min at room temperature. The proteins were then treated with 1:1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin as described under "Experimental Procedures." Controls included $\alpha_T^*$ that was untreated or $\alpha_T^*$ treated with a premixed aliquot of trypsin and soybean trypsin inhibitor. The proteolytic fragments were resolved by SDS-polyacrylamide gel (15%) electrophoresis. $\alpha_T^*$ in the GDP-bound, inactive conformation yielded an $\sim$23-kDa fragment, and $\alpha_T^*$ in the active conformation (GDP-AlF$_4^-$-bound) yielded an $\sim$32-kDa fragment following trypsin digestion. Lane 1, $\alpha_T^*$ treated with a premixed aliquot of trypsin and soybean tryptic inhibitor; lane 2, $\alpha_T^*$ treated with trypsin; lane 3, $\alpha_T^*$, preactivated with AlF$_4^-$ and then treated with trypsin; lane 4, untreated $\alpha_T^*$.

FIGURE 4. Trypsin proteolysis profiles for wild-type $\alpha_T^*$, $\alpha_T^*$(R174C), and $\alpha_T^*$(Q200L). The wild-type $\alpha_T^*$ subunit, $\alpha_T^*$(R174C), or $\alpha_T^*$(Q200L) (10 $\mu$g of each) was treated with or without AlF$_4^-$ for 30 min at room temperature. The proteins were then treated with 1:1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin as described under "Experimental Procedures." Controls included $\alpha_T^*$ that was untreated or $\alpha_T^*$ treated with a premixed aliquot of trypsin and soybean trypsin inhibitor. The proteolytic fragments were resolved by SDS-polyacrylamide gel (15%) electrophoresis. $\alpha_T^*$ in the GDP-bound, inactive conformation yielded an $\sim$23-kDa fragment, and $\alpha_T^*$ in the active conformation (GDP-AlF$_4^-$-bound) yielded an $\sim$32-kDa fragment following trypsin digestion. Lane 1, $\alpha_T^*$ treated with a premixed aliquot of trypsin and soybean tryptic inhibitor; lane 2, $\alpha_T^*$ treated with trypsin; lane 3, $\alpha_T^*$, preactivated with AlF$_4^-$ and then treated with trypsin; lane 4, untreated $\alpha_T^*$.

The $\alpha_T^*$(R174C) and $\alpha_T^*$(Q200L) Mutants Exhibit Measurable Rhodopsin-dependent Guanine Nucleotide Exchange Activity—Fig. 5 presents the results of $[^{35}S]$GTP$\gamma$S-binding assays, showing that a percentage of each of the $\alpha_T^*$ mutants can undergo R*-dependent GDP-GTP$\gamma$S exchange. The ability of wild-type $\alpha_T^*$ to exchange GDP for $[^{35}S]$GTP$\gamma$S exhibited the expected requirement for R* and the G$\beta$$\gamma$ complex (Fig. 5A). Specifically, the $\alpha_T^*$ subunit showed little detectable nucleotide exchange in the absence of R* and G$\beta$$\gamma$, similar to what has been observed for retinal $\alpha_T$, and exhibited only weak exchange activity when incubated with either R* or G$\beta$$\gamma$ alone. Both the $\alpha_T^*$(Q200L) (Fig. 5B) and $\alpha_T^*$(R174C) (Fig. 5C) mutants showed partial nucleotide exchange when assayed in the presence of R* and G$\beta$$\gamma$, such that ~40% of the total $\alpha_T^*$(Q200L) and 15% of the total $\alpha_T^*$(R174C) was capable of being activated. This would have been quite unexpected except for the findings in Fig. 4 that suggested a percentage of these mutants might be in the GDP-bound state and thus susceptible to R*- and G$\beta$$\gamma$-mediated nucleotide exchange.

Fig. 6 shows the results of R*-dependent nucleotide exchange activity as monitored by changes in Trp-207 fluorescence. Here again, wild-type $\alpha_T^*$ showed the expected requirement for R* and the G$\beta$$\gamma$ complex for GDP-GTP$\gamma$S exchange and the accompanying increase in Trp-207 fluorescence emission (Fig. 6A). The $\alpha_T^*$(Q200L) also showed an increase in Trp-207 fluorescence that was dependent on R* and G$\beta$$\gamma$. However, both the net increase as well as the rate of change for Trp-207 emission was reduced relative to the fluorescence changes accompanying the activation of wild-type $\alpha_T^*$ (Fig. 6B), similar to what we had seen when assaying GDP-$[^{35}S]$GTP$\gamma$S exchange (Fig. 5B). The changes in Trp-207 fluorescence for the $\alpha_T^*$(R174C) mutant were very difficult to detect (Fig. 6C), most likely because a lower percentage of this mutant was capable of responding to R*.

A Significant Percentage of the $\alpha_T^*$(R174C) Mutant as Well as the $\alpha_T^*$(Q200L) Mutant Is in the GDP-bound State—Fig. 7 shows the HPLC profiles obtained for wild-type $\alpha_T^*$, $\alpha_T^*$(Q200L), and $\alpha_T^*$(R174C) following denaturation of these purified recombinant proteins by boiling to release any prebound GDP or GTP. The bottom panel displays the control elution profiles for GDP and GTP. As expected, wild-type $\alpha_T^*$ only contained bound GDP and no detectable GTP. However, both the $\alpha_T^*$(R174C) and $\alpha_T^*$(Q200L) mutants, upon their isolation, exhibited heterogeneous populations of bound guanine nucleotide. Most interesting was the relatively high percentage of GDP-bound states for these mutants. Although both the $\alpha_T^*$(Q200L) and $\alpha_T^*$(R174C) mutants were isolated with bound GDP, we found that greater than 80% of the $\alpha_T^*$(Q200L) mutant and 50% of the $\alpha_T^*$(R174C) contained bound GDP.

Significant Percentages of Both the $\alpha_T^*$(R174C) and $\alpha_T^*$(Q200L) Mutants Contain Bound Pi—There have been reports indicating that Ga species containing both GDP and Pi are formed following GTP hydrolysis and may be capable of activating target/effectors (29–31). Given the results from HPLC analyses regarding the nucleotide occupancy of the R174C and Q200L mutants, we asked whether these...
mutants might exist as GDP-P_i complexes. A colorimetric assay for Pi was performed (see “Experimental Procedures”) to determine whether each of the mutants upon isolation contained bound Pi. The wild-type αT* subunit showed little or no detectable free Pi (i.e. typically less than 10% of the sample). However, both mutants appeared to contain significant amounts of Pi, i.e. 45% of the total αT*(Q200L) and nearly 40% of the total αT*(R174C). Fig. 8 summarizes the percentages for the different nucleotide-bound states, as determined from four preparations of wild-type αT* and each of the αT* mutants.

**DISCUSSION**

The GTPase reaction is an essential regulatory component of G protein-coupled signaling, as it ensures that signals are propagated for a finite rather than indefinite duration. A good deal of information regarding the underlying mechanisms for GTPase activity has come from the x-ray crystal structures for both small and large G proteins bound to aluminum fluoride (4–6). In the case of the large G proteins, the AlF_4^− species is associated with the GDP-bound Gα subunit, whereas for small G proteins, the GTPase-activating protein also needs to be bound, and often it is the AlF_3^− species that is present in the complex. However, in all cases, the aluminum fluoride-G protein interaction appears to mimic the transition state for the GTPase reaction. Based on this structural information, an essential arginine residue (termed the arginine finger) has been implicated as stabilizing the transition state. The essential arginine is present in the Switch I/linker region that connects to the large helical domain of Gα subunits but is contributed by the GTPase-activating proteins for small G proteins, which lack the helical domain. A second key residue implicated as being important for the GTPase reaction, is a highly conserved glutamine that is present in the Switch II domain of both large and small G proteins. It was originally suggested that the conserved glutamine residue was necessary for positioning water for its nucleophilic attack and to serve as a general base catalyst for the reaction. Although mutagenesis studies have verified that this residue is indeed necessary for full GTPase activity (32, 33), its exact role has been debated and in fact, more recently it has been argued that the γ-phosphate of GTP serves as the catalytic base in the reaction (17).

In the present study, we were interested in further examining the role of Gln-200 in the GTPase reaction of the recombinant αT*/αT_i1 chimera (αT*) and in comparing the effects of mutating this residue with substitutions of the arginine finger (Arg-174). We also wanted to see whether mutations of each of these residues yielded αT* subunits that were constitutively active in their abilities to stimulate PDE activity, and whether either mutant was still capable of responding to light-activated rhodopsin (R*) and Gα and undergoing stimulated GDP-GTP exchange. As expected, we found that both the R174C and Q200L mutations, when made in the αT* background, significantly impacted the GTPase reac-

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**FIGURE 6.** Rhodopsin-dependent GDP-GTP_S exchange activity as measured by changes in intrinsic tryptophan fluorescence. The wild-type αT* subunit (A), αT*(Q200L) (B), or αT*(R174C) (C), at a final concentration of 300 nM, was preincubated in the absence or presence of 300 nM GTP and 20 nM R* in HMDM buffer at room temperature, for 10 min. GTP-S (20 μM) was added and the fluorescent enhancement was measured (excitation = 300 nm, emission = 345 nm) until saturation.

**FIGURE 7.** HPLC measurement of the nucleotide occupancy of wild-type αT*, αT*(Q200L), and αT*(R174C). Wild-type αT* (top panel), αT*(Q200L) (second panel from top), or αT*(R174C) (second panel from bottom), at a final concentration of 100 μM, was boiled for 3 min and centrifuged at high speed for 10 min. An aliquot of the supernatant (20 μl) was injected into a C18 reverse phase HPLC column equilibrated with 100 mM KH_2PO_4, pH 6.5, 10 mM tetrabutylammonium bromide, 0.2% NaN_3, and 7.5% acetonitrile, and isocratic elution was performed. Elution of nucleotide standards GDP and GTP is indicated (bottom panel). The small peak with an elution time of 4.8 min is GMP.
tion, as assayed by measuring the release of $^{32}$P from $\alpha_\text{T}^*$ following GTP hydrolysis. Neither mutant was able to assume an AlF$_4^-$-induced transition state conformation as indicated by the lack of an enhancement of the Trp-207 emission that normally accompanies AlF$_4^-$ transition state conformation as indicated by the lack of an enhancement of these mutants to bind and respond to rhodopsin argued that they non-hydrolyzable GTP analogs or AlF$_4^-$ undergo the GTPase reaction and were consistent with the findings that R174C and Q200L mutants were compromised in their abilities to conformational changes in Switch II. These results verified that the APRIL 7, 2006•

Summary of the percentages of the different $\alpha_\text{T}^*$ subunits in the GTP-, GDP-, and GDP-Pi-bound states. The percentages of the different $\alpha_\text{T}^*$ subunits containing bound GTP and GDP were based on the HPLC analyses shown in Fig. 7. Also listed are the percentages of each $\alpha_\text{T}^*$ subunit containing bound P$_i$ ($\alpha_\text{T}^*$-GDP-P$_i$-complexes), based on phosphate analyses as described under "Experimental Procedures." Data points represent the average of 10 determinations by HPLC analysis and at least 5 determinations by phosphate analysis, using 4 different preparations of each of the recombinant proteins. The values for bound P$_i$ were corrected for the background obtained with buffer alone (usually ~3%).

| Mutant | GTP (HPLC) | GDP (HPLC) | GDP.Pi (P$_i$ assay) |
|--------|------------|------------|---------------------|
| Q200L  | 19 (+/- 1.4)% | 81 (+/- 1.4)% | 45 (+/- 6)% |
| R174C  | 47 (+/- 2.5)% | 53 (+/- 2.5)% | 37 (+/- 6)% |
| $\alpha_\text{T}^*$ | - | 100 % | 7 (+/- 4)% |

In light of the fact that the R174C and Q200L mutations resulted in the constitutive stimulation of the downstream effector enzyme (PDE), we are then led to conclude that for each of these mutants, all three nucleotide-bound states are signaling competent. This in turn would indicate that there are conformational states for Ga subunits that exist subsequent to GTP hydrolysis and are still capable of binding and regulating their target/effectors (Fig. 9).

The ability of the R174C and Q200L mutations to trap an $\alpha_\text{T}^*$-GDP-P$_i$ complex is consistent with earlier suggestions for the existence of this intermediate state, based on a pre-steady state kinetic analysis of GTP hydrolysis by transducin (29) and from studies characterizing different intermediates formed following the slow hydrolysis of GTP$\gamma$S by specific Ga$_\text{R}$ mutants (30, 31). The existence of a GDP-bound Ga conformation that is still capable of activating target/effectors and responding to G protein-coupled receptors like rhodopsin (i.e. Fig. 9, the $\alpha_\text{T}^*$-GDP species) would also explain some interesting but nonetheless puzzling results that we had earlier obtained with an $\alpha_\text{T}^*$ (E203A) mutant (34). This mutant was capable of undergoing rhodopsin-dependent GDP-GTP exchange and an accompanying GTP-dependent enhancement in Trp-207 fluorescence like wild-type retinal $\alpha_\text{T}^*$, but surprisingly, it was able to stimulate the PDE even when in the GDP-bound state. Taken together, the results obtained with the E203A mutant, and those described here for the R174C and Q200L mutants, also show that the conformational changes in Switch II, which are responsible for enhanced Trp-207 fluorescence and protection of Arg-204 against trypsin attack, can be uncoupled from the stimulation of effector (e.g. PDE) activity. We would expect that normally both of the conformational states that occur subsequent to GTP hydrolysis (i.e. Fig. 9, $\alpha_\text{T}^*$-GDP-P$_i$ and $\alpha_\text{T}^*$-GDP) are able to relax relatively quickly to the basal GDP-bound conformation that is signaling defective ($\alpha_\text{T}^*$-GDP). Apparently, the R174C and Q200L mutations slow the release of P$_i$ from the $\alpha_\text{T}^*$-GDP-P$_i$ species (i.e. Fig. 9, step 3), most likely by increasing the affinity of $\alpha_\text{T}^*$ for P$_i$, such that a significant amount of this species is still present upon the isolation of the recombinant proteins. These mutations also must significantly retard, if not effectively block, the conversion of a signaling competent but receptor-responsive $\alpha_\text{T}^*$-GDP species to the signaling inactive, receptor-responsive $\alpha_\text{T}^*$-GDP complex (step 4).

Overall, these findings hold some very interesting mechanistic implications. They demonstrate that both Arg-174 and Gln-200 not only are involved in the formation of the transition state for GTP hydrolysis but also influence the rate of P$_i$ release and set the limits for the lifetime of GTP hydrolysis occurring during the course of their preparation thus generating the GDP-bound species. In light of the fact that the R174C and Q200L mutations resulted in the constitutive stimulation of the downstream effector enzyme (PDE), we are then led to conclude that for each of these mutants, all three nucleotide-bound states are signaling competent. This in turn would indicate that there are conformational states for Ga subunits that exist subsequent to GTP hydrolysis and are still capable of binding and regulating their target/effectors (Fig. 9).

A scheme depicting the individual steps in the activation-deactivation cycle of the $\alpha$ subunit of transducin. Following rhodopsin and G$\beta$-$\gamma$-catalyzed GDP-GTP exchange (step 1), the $\alpha_\text{T}^*$ subunit undergoes a conformational change (depicted as $\alpha_\text{T}^*$-GTP) that enables it to bind and stimulate its downstream target/effector (the PDE). Following GTP hydrolysis (step 2), a $\alpha_\text{T}^*$-GDP-P$_i$ species is then converted to an $\alpha_\text{T}^*$-GDP species (step 3) that is still capable of stimulating the PDE but also is responsive to rhodopsin and can undergo GDP-GTP exchange. Normally, this state rapidly relaxes to the starting $\alpha_\text{T}^*$-GDP complex (step 4) that is signaling inactive. However, both the R174C and Q200L mutations slow step 3 and effectively block step 4. We would further suggest that the $\alpha_\text{T}^*$-GDP species functionally interacts with rhodopsin more slowly compared with the $\alpha_\text{T}^*$-GDP complex, as suggested by the slower rates for rhodopsin-stimulated GDP-GTP$\gamma$S exchange that were obtained with the R174C and Q200L mutants, compared with those measured for the wild-type protein.
the activated state for transducin. It may be that establishing the proper conformation for the transition state is not only necessary for ensuring an optimally regulated GTPase reaction but also ensures the efficient conversion between the subsequent conformational states that lead to the deactivation of the signal and reset the G protein switch so that it can respond to a new stimulatory event from its upstream activator. These findings also raise a number of interesting questions for future studies. For example, are the arginine finger and the conserved Switch II glutamine residue important for Pi release and the conversion of GDP-bound Gα subunits to signaling inactive states in other G proteins, how do target/effectors and RGS proteins influence these interconversions, and how does this compare with the mechanisms used by small G proteins?

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