Mechanism of GTP hydrolysis by G-protein α subunits

CHRISTIANE KLEUSS*, ANDRÉ S. RAW*, ETHAN LEE*, STEPHEN R. SPRANG†, AND ALFRED G. GILMAN*

*Department of Pharmacology and †Howard Hughes Medical Institute and Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235

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ABSTRACT Hydrolysis of GTP by a variety of guanine nucleotide-binding proteins is a crucial step for regulation of these biological switches. Mutations that impair the GTPase activity of certain heterotrimeric signal-transducing G proteins or of p21^{ras} cause tumors in man. A conserved glutamic residue in the α subunit of G proteins has been hypothesized to serve as a general base, thereby activating a water molecule for nucleophilic attack on GTP. The results of mutagenesis of this residue (Glu-207) in $G_{i\alpha 1}$ refute this hypothesis. Based on the structure of the complex of $G_{i\alpha 1}$ with GDP, Mg^{2+} , and AlF_4^- , which appears to resemble the transition state for GTP hydrolysis, we believe that Gln-204 of $G_{i\alpha 1}$, rather than Glu-207, supports catalysis of GTP hydrolysis by stabilization of the transition state.

Heterotrimeric G proteins play important roles as transducers and timers in a variety of intracellular signal transduction systems (1–5). G proteins are activated by binding of GTP in exchange for GDP. This reaction is catalyzed by interaction of the inactive, GDP-bound G protein with an appropriate agonist-bound receptor. Binding of GTP to the G protein causes conformational changes that result in dissociation of the GTP-liganded α subunit from both the receptor and a dimer of the G protein β and γ subunits. The activated α subunit and the $\beta\gamma$ dimer can then interact with and regulate the activities of downstream effectors, such as adenylyl cyclases, phospholipases, and ion channels.

G proteins deactivate themselves as a result of their intrinsic GTPase activity. The k_{cat} for this reaction is very slow, typically about 5 min⁻¹ for most members of the G_s and G_i subfamilies of G proteins (6–8). However, in some cases G protein-coupled effectors or other molecules can act as GTPase-activating proteins, increasing this rate to ≈ 50 min⁻¹ or greater (9). Mutations that impair the GTPase activity of $G_{s\alpha}$ and $G_{i\alpha}$ proteins have been associated with endocrine tumors, particularly of the pituitary (10). ADP-ribosylation of an arginine residue in $G_{s\alpha}$ by cholera toxin also inhibits GTPase activity (11). The result of this modification is irreversible activation of the G-protein α subunit with corresponding stimulation of its effector, adenylyl cyclase.

Detailed understanding of the mechanism of GTP hydrolysis by guanine nucleotide-binding proteins was, until recently, largely based on studies of p21^{ras} and translation elongation factor EF-Tu, for which high-resolution crystal structures are available (12–16). The guanine nucleotide-binding domains of these proteins are highly homologous to those of G-protein α subunits. Particularly vexing has been the role of Gln-61 in p21^{ras}. Although mutation of this residue drastically inhibits GTPase activity and is a common cause of human malignancies, elucidation of the structure of wild-type and mutant p21^{ras} proteins has not fully defined the role of this important residue. Mutations of the corresponding glutamine residue in heterotrimeric G proteins (Gln-204 in $G_{i\alpha 1}$; Gln-227 in $G_{s\alpha-L}$) also impair GTPase activity (17), as do mutations of

the arginine residue that is ADP-ribosylated by cholera toxin in $G_{s\alpha}$ (Arg-178 in $G_{i\alpha 1}$; Arg-201 in $G_{s\alpha-L}$) (18).

Noel *et al.* (19) have recently solved the structure of $G_{i\alpha 1}$, and Coleman *et al.* (32) have determined the structure of $G_{i\alpha 1}$. The structures of the guanosine 5'-[γ -thio]triphosphate (GTP γ S)-bound forms of these proteins [and of the Gln-204 $\rightarrow$ Leu (Q204L) mutant of $G_{i\alpha 1}$] again offer little insight into the role of the critical glutamine residue. However, Noel *et al.* (19) speculated that a glutamic residue (Glu-204 in $G_{i\alpha 1}$; Glu-207 in $G_{i\alpha 1}$) that is conserved in G-protein α subunits but corresponds to Tyr-64 in p21^{ras} could rotate so that its carboxylate side chain might deprotonate or polarize a crystallographically observed water molecule positioned for in-line attack on the γ -phosphate group of GTP. We have mutated Glu-207 in $G_{i\alpha 1}$ to glutamine or alanine and characterized the altered proteins in an attempt to confirm or refute the model of GTP hydrolysis proposed by Noel *et al.*

MATERIALS AND METHODS

Plasmids and Proteins. A cDNA containing the entire coding sequence of $G_{i\alpha 1}$ (20) was ligated into the *Eco*RI site of M13mp19. Mutagenesis was performed by the method of Kunkel *et al.* (21), using 5'-CCACTTCTTCCGCTGC-GATCGTTGGCCTCCCACG-3' as the mutagenic oligonucleotide to generate E207Q $G_{i\alpha 1}$ and 5'-CCACTTCTTC-GCGCAGATCTCTGGCC-3' to generate E207A $G_{i\alpha 1}$. The mutations were confirmed by DNA sequencing. The complete coding sequences (*Nco* I–*Eco*RI fragments) of the two mutants and the wild-type protein were subcloned into the *Nco* I and *Sma* I sites of plasmid H₆pQE60 (22) to generate cDNAs encoding proteins with hexahistidine sequences at their amino termini. These plasmids were transformed together with plasmid pREP4 into *Escherichia coli* BL21(DE3). Proteins were expressed and purified on Ni²⁺-NTA resin as described (22); the mutations were confirmed by protein sequencing. Mutants R178C $G_{i\alpha 1}$ and Q204L $G_{i\alpha 1}$ (not histidine-tagged) were generated, expressed, and purified as described (22, 23). The sequences of these mutants were confirmed by DNA sequencing, and their crystal structures have been solved.

Trypsinization of $G_{i\alpha 1}$. Wild-type or mutant $G_{i\alpha 1}$ protein (1 mg/ml) was incubated for 150 min at 30°C in 50 mM Na-Hepes, pH 8.0/1 mM EDTA/5 mM dithiothreitol/10 mM MgSO₄ plus either 100 μ M concentrations of GDP, GTP, or GTP γ S or 30 μ M AlCl₃ and 10 mM NaF. Trypsin was added (26 μ g/ml) as indicated. After 10 min at 30°C, sample buffer was added and samples were boiled.

GTP γ S Binding Kinetics. Proteins (10 μ g/ml) were incubated at 30°C in 100 mM Na-Hepes, pH 8.0/1 mM EDTA/10 mM dithiothreitol/10 mM MgSO₄/2 μ M [³⁵S]GTP γ S (700 cpm/pmol). Aliquots (100 μ l) were withdrawn at the indicated times, added to 2 ml of ice-cold wash buffer (20 mM Tris-HCl, pH 8.0/100 mM NaCl/25 mM MgCl₂), and analyzed as described (24). Values of k_{app} for the binding reaction

were calculated by a nonlinear least-squares fit to the equation $B = B_{eq}(1 - e^{-kt})$.

Steady-State Hydrolysis of GTP. Proteins (120 nM) were incubated at 30°C in 50 mM NaHepes, pH 8.0/1 mM EDTA/5 mM dithiothreitol/10 mM MgSO₄/5 μM [γ -³²P]GTP (3200 cpm/pmol). Aliquots (50 μl) were removed at the indicated times and [³²P]P_i was determined as described (6).

Steady-State Binding of GTPγS and GTP. Proteins (150 nM) were incubated for 3 hr at 30°C in 100 mM NaHepes, pH 8.0/1 mM EDTA/10 mM dithiothreitol/10 mM MgSO₄ with 2 μM [³⁵S]GTPγS (700 cpm/pmol) or 2 μM [γ -³²P]GTP (3300 cpm/pmol). The reaction was quenched by addition of ice-cold wash buffer and analyzed as described (24).

Determination of k_{cat} for Hydrolysis of GTP. Proteins (425 nM) were incubated for 15 min at 30°C and then for 5 min at 20°C in 50 mM NaHepes, pH 8.0/10 mM EDTA/5 mM dithiothreitol/2 μM [γ -³²P]GTP (7300 cpm/pmol). Reactions (at 20°C) were started by addition of MgSO₄ and GTPγS to final concentrations of 20 mM and 200 μM, respectively. Aliquots (50 μl) were analyzed for [³²P]P_i at the indicated times as described (6).

RESULTS

Protein Purification. Wild-type G_{ia1} and two mutants (E207Q and E207A) were expressed with six histidine residues preceding the initiator methionine of G_{ia1} and were purified in one step using Ni²⁺-NTA agarose. The Q204L and R178C mutants of G_{ia1} did not contain amino-terminal histidine residues and were purified conventionally. In each case ≈40 mg of highly purified protein was obtained from a 1-liter bacterial culture (Fig. 1, lanes 1, 6, 11, 16, and 21). Addition of histidine residues at the amino terminus does not alter the kinetics of guanine nucleotide binding or hydrolysis by G_{ia1}.

Trypsinization. Certain conformational changes that accompany activation of G-protein α subunits by GTP are detectable by enhancement of intrinsic tryptophan fluorescence (25) or acquisition of resistance to proteolysis by trypsin (26). GDP-bound α subunits are readily hydrolyzed by trypsin to relatively small fragments; GTP- or GTPγS-activated forms of these polypeptides are rapidly cleaved near the amino terminus, and 37- to 39-kDa products then accumulate. When wild-type G-protein α subunits are incubated with GTP, the GDP-bound form of the protein predominates because the rate of hydrolysis of GTP greatly exceeds the rate of release of product (GDP). Wild-type G_{ia1} and the E207Q and E207A mutants are protected from proteolysis after incubation with GTPγS but not with GTP, indicating the likelihood that these proteins all have significant GTPase activity (Fig. 1). In contrast, the GTPase-deficient proteins R178C G_{ia1} and Q204L G_{ia1} are protected following incubation with either GTPγS or GTP. R178C G_{ia1} appears to have a GTPase activity that is slightly higher than

that of Q204L G_{ia1}, since the amount of protein protected after incubation with GTP is lower for the R178C mutant (Fig. 1, lane 18).

AlF₄⁻ and Mg²⁺ bind to the GDP-bound form of G-protein α subunits and cause many of the changes seen with nonhydrolyzable analogs of GTP, including protection from tryptic proteolysis (Fig. 1, lane 5) (27). It is generally believed that the AlF₄⁻ complex mimics the γ-phosphate of GTP and thus activates G-protein α subunits in concert with GDP (28, 29). Of interest, Q204L G_{ia1} is completely degraded by trypsin in the presence of AlF₄⁻, suggesting that AlF₄⁻ does not bind to or activate the mutant protein. The crystal structure of the complex of AlF₄⁻ with GDP-G_{ia1} reveals interactions between Gln-204 and the AlF₄⁻ ligand that are consistent with these possibilities (see below).

Guanine Nucleotide Binding and Hydrolysis. All of the proteins used in these studies bound GTPγS to a similar extent at equilibrium (usually about 0.7 mol/mol of protein). The rate of binding of GTPγS to these proteins is limited by the rate of dissociation of GDP; this value for wild-type G_{ia1} ($k_{app} = 0.027 \text{ min}^{-1}$) was essentially identical to that reported earlier for the non-histidine-tagged protein (7) and to the rates determined for the E207Q and E207A mutants (Fig. 2). Similar rates of GTPγS binding were also observed for R178C G_{ia1} (Fig. 2) and for the Q204L mutant (data not shown). The rates of dissociation of GTPγS from all five proteins were exceedingly slow (data not shown).

The steady-state rate of GTP hydrolysis by the five proteins is shown in Fig. 3. This rate is equal to the rate of GTPγS binding (and GDP dissociation) for the wild type and Glu-207 mutants, while it is substantially reduced for R178C and Q204L G_{ia1}. Thus, steady-state GTP hydrolysis is limited by GDP dissociation for the wild-type protein and the Glu-207 mutants, while the k_{cat} for GTP hydrolysis by R178C or Q204L G_{ia1} is slower than the rate of product dissociation and substrate binding.

The fractional steady-state occupancy of the guanine nucleotide-binding site of a G-protein α subunit by GTP (compared with GTPγS) is given by the expression $F(\text{GTP}) = k_{diss,GDP}/(k_{cat,GTP} + k_{diss,GDP})$. Thus, if k_{cat} is high relative to $k_{diss,GDP}$, the G protein exists largely in the GDP-bound form at steady state. Binding of [γ -³²P]GTP to the five proteins was compared with that of [³⁵S]GTPγS after a 3-hr incubation (Fig. 4). Fractional occupancies by [γ -³²P]GTP for E207Q G_{ia1} and E207A G_{ia1} (0.012% and 0.014%) were only slightly greater than the value for the wild-type protein (none detected), indicating an insignificant difference in k_{cat} values among these three proteins. The high fractional occupancies by GTP for R178C and Q204L G_{ia1} are consistent with previous estimates of the effects of these mutations in G_{sa} (17, 18). The value of k_{cat} for R178C G_{ia1} calculated from the data of Fig. 4 is 0.014 min^{-1} , which is >100-fold lower than the value observed for wild-type G_{ia1} (Fig. 5 and ref. 7).

Fig. 1. Trypsinization of wild-type and mutant forms of G_{ia1}. Wild-type or mutant G_{ia1} was incubated with either GDP, GTP, GTPγS, or AlCl₃/MgCl₂/NaF (AMF) and trypsin where indicated. Proteins were separated in a sodium dodecyl sulfate/11% polyacrylamide gel and stained with Coomassie blue. Lanes 1–5, wild-type G_{ia1}; lanes 6–10, E207A G_{ia1}; lanes 11–15, E207Q G_{ia1}; lanes 16–20, R178C G_{ia1}; lanes 21–25, Q204L G_{ia1}.

FIG. 2. $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding kinetics. Proteins were treated as described in *Materials and Methods*. Data are normalized to binding after 180 min. Maximal binding according to amount of protein was 58% for wild-type (WT) $G_{i\alpha 1}$, 76% for E207A $G_{i\alpha 1}$, 70% for E207Q $G_{i\alpha 1}$, 65% for R178C $G_{i\alpha 1}$, and 70% for Q204L $G_{i\alpha 1}$. Values of k_{app} were calculated by a nonlinear least-squares fit to the equation $B = B_{eq}(1 - e^{-kt})$.

Values for k_{cat} can be calculated directly by observation of a single round of GTP hydrolysis. This is accomplished (Fig. 5) by allowing the protein to bind GTP in the absence of Mg^{2+} . Catalysis is then initiated by addition of Mg^{2+} , and rebinding of radiolabeled substrate is prevented by addition of unlabeled guanine nucleotide. These experiments indicate k_{cat} values of 3.4 min^{-1} , 1.1 min^{-1} , and 2.2 min^{-1} for wild-type $G_{i\alpha 1}$, E207A $G_{i\alpha 1}$, and E207Q $G_{i\alpha 1}$, respectively. R178C $G_{i\alpha 1}$ and Q204L $G_{i\alpha 1}$ did not hydrolyze detectable amounts of GTP over the short time frame of this experiment.

DISCUSSION

A water molecule is visualized in the crystal structures of $G_{i\alpha}$ and $G_{i\alpha 1}$ (as well as in p21^{ras} and EF-Tu) that is ideally positioned for in-line nucleophilic attack on the γ -phosphate group of GTP. Noel *et al.* (19) have suggested that rotation of the side chain of Glu-207 would bring the carboxylate group of this residue into position to act as a general base, deprotonating and activating the water molecule. Alternatively, the carboxylate group could polarize the water molecule or

FIG. 3. Steady-state hydrolysis of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$. Proteins were treated as described in *Materials and Methods*. Aliquots (50 μL) were removed at the time points indicated and $[\text{P}^{32}]\text{P}_i$ was determined.

FIG. 4. Steady-state binding of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and $[\text{S}^{35}]\text{GTP}\gamma\text{S}$. Fractional occupancy is expressed as binding of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ as a percentage of that observed for $[\text{S}^{35}]\text{GTP}\gamma\text{S}$.

stabilize positive charge developing on the water molecule in the transition state. If any of these mechanisms was operative, mutation of Glu-207 to glutamine should slow the rate of GTP hydrolysis and mutation to alanine should have a substantial deleterious effect. The biochemical data reported here are not consistent with a role for Glu-207 in GTP hydrolysis, leaving moot the question of the conservation of this residue among all G-protein α subunits. The corresponding residue in p21^{ras}, Tyr-64, does appear to be necessary for GTPase activity, although hydrophobic substitutions are tolerated (30).

Coleman *et al.* (32) have recently solved the structure of the complex of $G_{i\alpha 1}$ with $\text{GTP}\gamma\text{S}$. In this structure (and that of $\text{GTP}\gamma\text{S}-G_{i\alpha}$), Gln-204 is close to the putative nucleophilic water molecule. A small rotation would place the carboxamide side chain adjacent to the water. The structure of Q204L $G_{i\alpha 1}$ has also been solved and reveals no significant perturbations, suggesting that the explanation for the impaired GTPase activity of this mutant lies with the chemistry of the side-chain substitution rather than with any conformational or steric effects. Although mutation of Gln-61 in p21^{ras} to glutamic acid was originally thought to impair GTPase ac-

FIG. 5. Determination of k_{cat} for the hydrolysis of GTP. After 21 min, MgSO_4 and $\text{GTP}\gamma\text{S}$ were added to final concentrations of 20 mM and 200 μM , respectively. Aliquots (50 μL) were analyzed for $[\text{P}^{32}]\text{P}_i$ at the indicated times.

tivity, it is now known that the k_{cat} for this mutant is increased 20-fold, although its K_m for GTP is, not surprisingly, much higher (31). Thus, this observation is also consistent with the view that Gln-61 plays a distinct role in catalysis. This mutation demonstrates that placement of a base at position 61 can accelerate catalysis, presumably by activation or stabilization of the nucleophilic water molecule, but it does not prove that this is the role normally played by glutamine at this position. Finally, Coleman *et al.* (32) have determined the structure of $G_{i\alpha 1}$ complexed with GDP, AlF_4^- , and Mg^{2+} . Here, the Al^{3+} assumes a six-coordinate octahedral coordination sphere with its four fluorine atoms lying in one plane; the β -phosphate oxygen atom and the presumptive nucleophilic water molecule lie trans to each other. Thus, this complex appears to mimic a structure approaching the transition state for GTP hydrolysis. Significantly, Gln-204 has moved from its ground-state position in the GTP γ S structure to a position in which its carboxamide side chain is within hydrogen-bonding distance of both the nucleophilic water molecule and a fluorine atom. Glu-207 has not moved in this structure. We thus support the model (for both G-protein α subunits and p21^{ras}) where Gln-204 ($G_{i\alpha 1}$) or Gln-61 (p21^{ras}) supports catalysis of GTP hydrolysis by stabilization of the transition state. This may include polarization and orientation of the attacking water molecule and/or stabilization of developing charge in the transition-state complex.

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