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GTP analogue hydrolysis by the Gs protein: implication for the role of catalytic glutamine in the GTPase reaction

Tsaffrir Zor[1,a], Ronit Andorn[a], Ilya Sofer[a], Michael Chorev[b], Zvi Selinger[a,*]

[a]*Department of Biological Chemistry and the Kuhne Minerva Center for Studies of Visual Transduction, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*

[b]*Department of Pharmaceutical Chemistry and the Kuhne Minerva Center for Studies of Visual Transduction, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*

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Abstract Hydrolysis of GTP, bound to members of the G-protein superfamily, terminates their downstream signaling activity. A conserved glutamine serves a critical role in this pivotal guanosine triphosphatase (GTPase) reaction. However, the role of the catalytic glutamine in GTP hydrolysis is still not well understood. We have employed substrate-assisted catalysis to probe the catalytic mechanism of $G_s\alpha$ using GTP analogues. These GTP analogues, each having different functional groups, were designed to support or refute particular putative GTPase mechanisms. We have found that a hydrogen donor group, in close proximity to the γ-phosphate of GTP, is necessary and sufficient to substitute for the function of the catalytic glutamine in the GTPase reaction.

Key words: GTP hydrolysis; GTP binding protein; cAMP; Substrate-assisted catalysis

1. Introduction

Heterotrimeric G-proteins are molecular transducers and timers that mediate signal transduction. They are active in the GTP-bound form, while hydrolysis of the bound GTP is a common reaction that converts these proteins from the active into the inactive state [1]. This GTPase reaction underlies the regulatory cycle of G-proteins [2] and is central to their physiological function. Experiments in vitro showed that a conserved glutamine is essential for the GTPase activity of both small [3] and heterotrimeric G-proteins [4,5]. Mutation of Gln-61 of the Ras protein is commonly associated with oncogenic transformation [6]. Similarly, mutation of the corresponding Gln-227 of $G_s\alpha$ was found in thyroid and pituitary tumors [7,8]. Structural studies of Gα subunits complexed with the transition state analogue GDP-AlF_4^- have implicated the catalytic glutamine in transition state formation and stabilization [9,10]. While this essential GTPase reaction has been extensively studied, the exact role of the conserved glutamine in the catalytic process has not been unequivocally determined.

Generally, GTP analogues with substituents attached to the γ-phosphate, either through phosphonoamidate or ester linkage are not hydrolyzed by G-proteins [11–15]. We have recently shown, however, that the GTP analogue 3,4-diaminobenzophenone phosphonoamidate-GTP (DABP-GTP, analogue **8** in Fig. 1) is hydrolyzed at the same rate as GTP, both by the wild-type $G_s\alpha$ and by the cholera toxin modified $G_s\alpha$ [11]. Moreover, DABP-GTP was able to rescue the GTPase-deficient Gln-227Leu $G_s\alpha$ mutant. We have suggested that the rescue involves a mechanism of substrate-assisted catalysis. In this mechanism, a mutant inactive enzyme, lacking a catalytic amino acid, is active on a substrate bearing the essential functional groups that have been deleted by the mutation. Modeling of DABP-GTP [11], superimposed on the crystal structure of transducin α-GDP-AlF_4^- complex [9], suggests that in the wild type $G_s\alpha$, the catalytic glutamine is significantly displaced by the bulky modification on the γ-phosphate while the other catalytic interactions are preserved. The model also predicts that the carbonyl of the benzophenone points away from the active site and therefore is not part of the catalytic machinery.

In the present work, we have prepared a series of GTP analogues carrying different substituents on the γ-phosphate. Their resistance or susceptibility to hydrolysis by the $G_s\alpha$ subunit revealed the functional requirements for hydrolysis of phosphonoamidate analogues of GTP. These studies can be used to describe the mechanism of GTP hydrolysis. It is concluded that a hydrogen donor group must be present in close proximity to the γ-phosphate of GTP. In the case of GTP, this function is carried out by the catalytic glutamine, whereas in the GTP analogues this function is fulfilled by the modification which bears free amino or hydroxyl groups.

2. Materials and methods

2.1. Materials

[α-^{32}P]ATP was from NEN, alkaline phosphatase was from Boehringer Mannheim, *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide-HCl (referred to as the carbodiimide) was from Fluka, Na_2H_2GTP was from Pharma-Waldhof and 1,4-dioxane was from Mallinckrodt. The following aniline derivatives were purchased from Aldrich and used for the synthesis of GTP analogues **3**, **4**, **5**, **6**, **7**, **9**, **10** and **11** respectively: 1,3-phenylenediamine, aniline, 2-fluoroaniline, 2-toluidine, 2-anisidine, 1,2-phenylenediamine, 4-nitro-1,2-phenylenediamine and 2-aminophenol.

2.2. Preparation of GTP analogues

Na_2H_2GTP (20 μmol, 1 eq) and the carbodiimide (100 μmol, 5 eq) were dissolved in 0.5 ml of 0.125 M MES buffer, pH 6.8. The aniline derivative (200 μmol, 10 eq), dissolved in 0.2 ml of 1,4-dioxane, was added and the mixture was kept at room temperature, under inert gas, in the dark. The dioxane used for the synthesis of compounds **3**, **4**, **5**, **9** and **11** was peroxide-free (prepared by passage through aluminium

*Corresponding author. Fax: (972) (2) 6527427.
E-mail: selinger@vms.huji.ac.il

[1]The first two authors contributed equally to this work.

Abbreviations: DABP-GTP, 3,4-diaminobenzophenone phosphonoamidate-GTP; MABP-GTP, monoaminobenzophenone phosphonoamidate-GTP; m-AcAGTP, *meta*-acetylanilidoguanosine 5'-triphosphate; Gpp(NH)p, guanosine-5'-(β,γ-imido)triphosphate; G-proteins, guanine nucleotide binding proteins

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oxide column). DMSO was used for the synthesis of compound **10** instead of dioxane. Reaction time was 4 h, except for compounds **3**, **6** and **10** (14 h). The quantities of the aniline derivative and the carbodiimide used for the synthesis of compound **10** were reduced 2-fold. Dioxane and excess aniline derivative were extracted with ethyl acetate and the resulting water phase was applied to a preparative C-8 RP-HPLC column (250×10 mm). The product-containing fractions were subsequently lyophilized and dissolved in 50 mM MOPS buffer (pH 7.35). The yields were 70–95% for all GTP analogues except for **10** (15%) and **11** (30%). To eliminate interference by contamination of unmodified guanine nucleotides in the biochemical assays, the solution was treated with 14 units of alkaline phosphatase for 10 min at 30°C. The enzyme was removed by centrifugation in Centricon-30 (Amicon) and the filtrate was stored under inert gas, at −70°C. These GTP analogues were stable for at least 3 months. The synthesis of GTP analogues **1** [12], **2** and **8** [11] was previously described. The identity of each purified GTP analogue was verified by acidic hydrolysis of the acid-labile phosphorus-nitrogen bond. The hydrolysis products coeluted in HPLC with the respective aniline derivatives that served as the starting compound.

2.3. Assay of adenylyl cyclase activity

Rat parotid membranes were prepared as previously described [16]. The ability of GTP analogues to persistently activate the adenylyl cyclase was determined as described by Cassel and Selinger [17]. The GTP analogue (2 μM) was incubated with the membranes for 5 min at 30°C in the presence of 50 μM D,L-epinephrine in medium containing 50 mM MOPS buffer (pH 7.4), 3.3 mM $MgCl_2$, 0.11 mM EGTA and 2 mM 2-mercaptoethanol. Subsequently, adenylyl cyclase activity was assayed in the presence of 50 μM propranolol at 30°C for 10 min. The adenylyl cyclase reaction was initiated by the addition of 0.3 mM ATP and [α-^{32}P]ATP in a reaction medium containing the previously mentioned materials and 1 mM cAMP, 0.5 mM 3-isobutyl-1-methylxanthine, 15 mM creatine phosphate and 5 U creatine phosphokinase. The final volume was 0.1 ml. The reaction was terminated by the addition of 1.25% SDS and cAMP was measured according to the method of Salomon et al. [18].

2.4. Protein determination

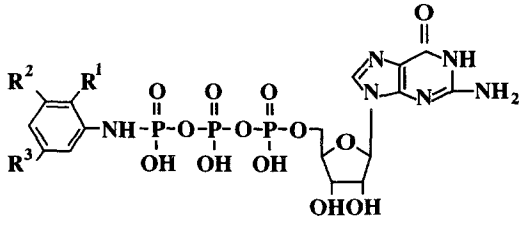
Protein was determined by a modified Bradford procedure, which gives linear results [19], using bovine serum albumin as standard.

3. Results and discussion

Heterotrimeric G-proteins are key components of signal transduction [1]. An activated receptor turns the G-protein on by facilitating the exchange of bound GDP for free GTP [17]. The GTP-bound G-protein, in turn, transmits the signal by activating a downstream effector. A GTPase reaction turns the process off by converting the bound GTP to GDP [2]. In the adenylyl cyclase system, activation of the effector by GTP depends on the continuous presence of the agonist. In contrast, activation by hydrolysis-resistant GTP analogues requires the presence of the agonist only initially to promote formation of the complex between the G-protein and the GTP analogue. Binding of these analogues to the G-protein results in persistent activation of the effector even after the receptor has been blocked by an antagonist [20]. Persistent activation of the adenylyl cyclase is therefore an indication for the resistance of GTP analogues to hydrolysis.

We have previously shown that the GTP analogue DABP-GTP (analogue **8** in Fig. 1) is hydrolyzed by the glutamine-mutant Gln-227Leu $G_s\alpha$, at a rate similar to that of the unmodified $G_s\alpha$ (0.11 s^{-1}) while both GTP and the GTP analogue monoaminobenzophenone phosphonoamidate-GTP (MABP-GTP, analogue **2** in Fig. 1) were not hydrolyzed by this mutant [11]. These results, as well as the computer model [11] suggest that the conserved glutamine of the native $G_s\alpha$, which is involved in GTP hydrolysis, does not take part in the enzymatic hydrolysis of DABP-GTP. It can therefore be assumed that the enzymatic hydrolysis of analogues **8–11** (Fig. 1) is assisted by the modified substrates and not by the catalytic glutamine.

To gain further insight into the mechanism of substrate-assisted catalysis we have prepared a series of GTP analogues (Fig. 1) and tested whether they are hydrolyzed by the adenylyl cyclase system. Each of these analogues, bearing a different modification on the γ-phosphate of GTP, was designed to test a particular putative mechanism of hydrolysis. For compounds **1–7** (Fig. 1), we found that activation of adenylyl cyclase by the analogues, in the continuous presence of the agonist, was similar in magnitude to the persistent activation in the presence of antagonist (Fig. 2), indicating that these analogues are hydrolysis-resistant. In contrast, GTP and analogues **8–11** (Fig. 1) did not persistently activate the adenylyl cyclase (Fig. 2), indicating that they undergo hydrolysis. These analogues clearly caused activation of adenylyl cyclase in the presence of an agonist (Fig. 2). They also efficiently inhibited the persistent activation of adenylyl cyclase caused by Gpp(NH)p (not shown). Taken together, these findings indicate that, like GTP itself, GTP analogues **8–11** bind to the $G_s\alpha$ subunit and subsequently undergo hydrolysis. In the case of DABP-GTP, the cleavage of the radiolabeled analogue showed that the absence of persistent adenylyl cyclase activity is due to enzymatic hydrolysis of the GTP analogue [11]



Compound	R^1	R^2	R^3	Hydrolysis
(m-AcAGTP) 1	H	H	$-C(=O)-CH_3$	
(MABP-GTP) 2	H	H	$-C(=O)-C_6H_5$	
3	H	NH_2	H	
4	H	H	H	−
5	F	H	H	
6	CH_3	H	H	
7	OCH_3	H	H	
(DABP-GTP) 8	NH_2	H	$-C(=O)-C_6H_5$	
9	NH_2	H	H	
10	NH_2	H	NO_2	+
11	OH	H	H	

Fig. 1. Chemical structure of the GTP analogues.

rather than due to spontaneous hydrolysis or dissociation of the analogue from the G-protein.

The presence of an *ortho* amino group in analogue **8** (DABP-GTP) is crucial to overcome the hydrolysis resistance evident in analogues **1** (m-AcAGTP) and **2** (MABP-GTP) ([11] and Fig. 2). Analogue **8** (DABP-GTP) carries both the amino and carbonyl functional groups of the glutamine side chain. It was therefore assumed that both the amine and the carbonyl are necessary for substitution of the glutamine function. However, the structural model [11] predicted that the carbonyl in the benzophenone moiety points away from the active site and therefore is not part of the catalytic machinery. This prediction is corroborated by the finding that compound **9**, a similar GTP analogue which lacks the carbonyl group, undergoes hydrolysis (Fig. 2). A tautomer-based catalytic mechanism has been suggested [9], in which the glutamine, through both the amino and the carbonyl groups, mediates the transfer of a proton from the nucleophilic water molecule to the γ-phosphate. While this is theoretically possible for analogue **8** (DABP-GTP), this is consistent neither with the computer model [11] nor with the finding that GTP analogue **9**, which lacks the carbonyl group, undergoes hydrolysis.

There are several other mechanisms by which DABP-GTP can undergo hydrolysis: the free amino group could serve as the general base for the nucleophilic water molecule (Fig. 3a) or act as a nucleophile by itself (Fig. 3b). An aromatic amine is a weak base and a poor nucleophile. However, the phosphonoamidate nitrogen contributes by resonance to the relative negative charge on the free amino group and increases its basic and nucleophilic character. A hydrogen bond with the phosphonoamidate nitrogen would also increase these properties. Analogue **10** was synthesized to test the importance for catalysis of the basic or nucleophilic character of the amino group. A powerful electron withdrawing nitro group, in a *para* position to the free amine, should diminish these properties and thereby make analogue **10** hydrolysis-resistant. The finding that analogue **10** undergoes hydrolysis is not consistent with the above mechanisms.

The free amino group could act as an electron donor that confers a general base property on the phosphonoamidate nitrogen, either by resonance or by acting as an acceptor in a hydrogen bond formation (Fig. 3c). In this case, however, analogue **5** should also be efficiently hydrolyzed, since fluoride is also a hydrogen acceptor. The finding that analogue **5** is hydrolysis-resistant argues against this mechanism. The ability of analogue **11** to undergo hydrolysis indicates that the amino group could be replaced by a hydroxyl. The hydroxyl group could act either as an electron donor or as a hydrogen donor. Replacement of the hydroxyl by a methoxy group in analogue **7** discriminates between these two actions. The methoxy is a similar electron donor but confers hydrolysis resistance on the GTP analogue. These findings indicate that it is unlikely that the amino group serves as an electron donor. The remaining mechanism is that analogue **11** and analogue **9** undergo hydrolysis because both the amino and the hydroxyl groups participate in a hydrogen bond formation as hydrogen donors (Fig. 3d). As expected, analogue **4** with an unsubstituted *ortho* position and analogue **6** with an *ortho* methyl group are both hydrolysis-resistant as neither can serve as a hydrogen donor. The position of the hydrogen donor group is critical as analogue **3**, in which the amino group was transferred from the *ortho* to the *meta* position, is hydrolysis-resistant.

Structural studies of G_t and G_i α-subunits with GDP complexed with AlF_4^-, which mimics the transition state of the GTPase reaction, showed that the catalytic glutamine residue (Gln-200 in $G_t\alpha$ and Gln-204 in $G_i\alpha$) contacts the water molecule and one of the fluorides of AlF_4^- [9,10]. It was therefore suggested that the catalytic glutamine stabilizes the transition state by hydrogen bonding to the nucleophilic OH^- and to planar phosphate oxygens.

In an attempt to draw conclusions from the mechanism of analogue hydrolysis to that of GTP, we assume that both substrates are cleaved by the same mechanism even though the analogues are, formally, phosphate diesters and the γ-phosphate of GTP a phosphate monoester, thus differing in the number of negative charges. With that assumption, our results that a hydrogen donor group is both necessary and sufficient for hydrolysis of the GTP analogues are consistent

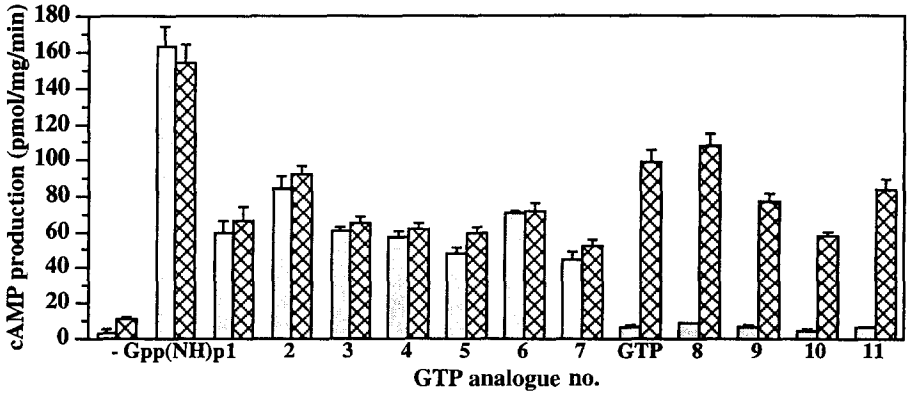
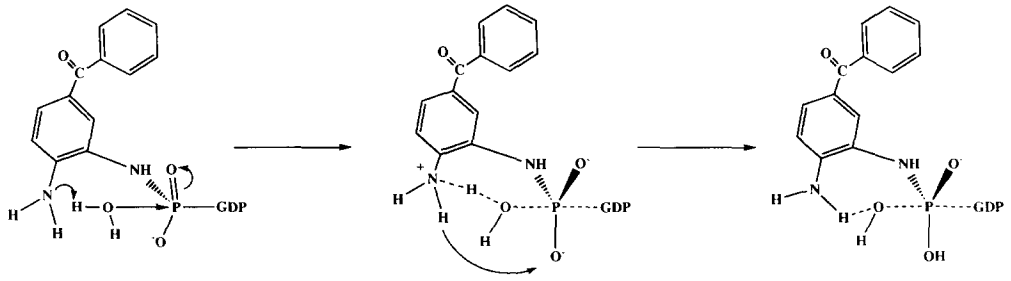
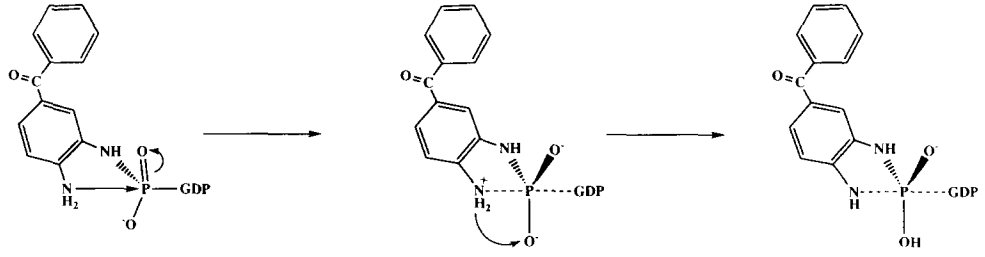


Fig. 2. Activation of adenylyl cyclase by GTP analogues. Persistent activation of the adenylyl cyclase (dotted bars) was tested, as described in Section 2. Assay of persistent activation was carried out by incubation of rat parotid membranes (15 μg) with the GTP analogues (2 μM) in the presence of the β-adrenergic agonist epinephrine (50 μM) at 30°C for 5 min, followed by an assay of adenylyl cyclase activity, carried at 30°C for 10 min, in the presence of the β-adrenergic antagonist, propranolol (50 μM). During the first incubation period, the activated β-adrenergic receptor charges the G-protein with the GTP analogue, while the subsequent assay of adenylyl cyclase activity in the presence of the β-adrenergic antagonist, propranolol, measures whether the G-protein remains persistently active. Adenylyl cyclase activity in presence of epinephrine (crossed bars) was assayed as described for persistent activation but without addition of propranolol. The assay was performed in triplicate and the experiment was repeated at least five times for each GTP analogue.

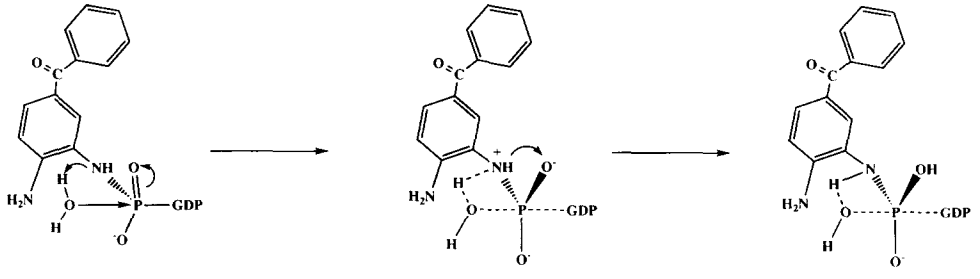
a. The free amino group acts as a base:



b. The free amino group acts as a nucleophile:



c. The phosphonamide acts as a base:



d. The free amino group stabilizes the transition state:

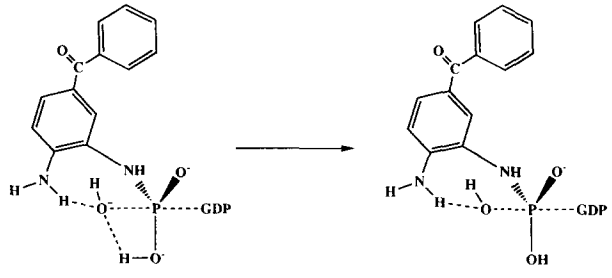


Fig. 3. Schematic representation of putative intermediate states of DABP-GTP hydrolysis. a: The free amino group of DABP-GTP could act as a base and mediate the transfer of a proton from the attacking water molecule to the γ-phosphate oxygen. b: The free amino group of DABP-GTP could act as a nucleophile, attack the γ-phosphate and transfer a proton to the γ-phosphate oxygen. c: The Phosphonoamidate nitrogen could act as a base and mediate the transfer of a proton from the attacking water molecule to the γ-phosphate oxygen. d: The free amino group could stabilize the pentavalent intermediate through a hydrogen bond. This is a schematic representation, bond angles and distances are arbitrary and do not represent actual coordinates. For technical reasons, both hydrogen bonds and bonds that are formed and broken down during the hydrolytic reaction are shown as dashed lines.

with the following model: the hydrogen donor function of the amino group participates in GTP analogues hydrolysis by stabilizing the transition state through hydrogen bonding (Fig. 3d). Our functional studies complement the structural studies of GDP–AlF_4^- complexes of α subunits, suggesting that in the case of GTP the catalytic glutamine participates in the GTPase reaction by a similar mechanism of transition state stabilization.

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