# TOPOLOGY OF THE GTP-BINDING SITE OF ADENYLYL CYCLASE FROM PIGEON ERYTHROCYTES

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## 1. Introduction

Hormone-activated adenylyl cyclases of higher organisms are modulated by nucleotides mainly GTP [1–6]. Derivatives of GTP like Gpp(NH)p, Gpp($CH_2$)p and GTPγS, which are not or more slowly hydrolized than GTP are more potent activators than GTP itself. In some instances, for example in avian erythrocyte membranes the firm binding of the analogs results in quasi irreversible activation of adenylyl cyclase. Pfeuffer and Helmreich have suggested that the nucleotide binding protein exhibits GTPase activity and that the metabolically stable guanylyl analogs may be visualized as transition-state intermediates which would explain tight binding and irreversible activation [5]. But in order to verify this hypothesis it will be necessary, to purify and characterize the GTPase activity because membranes contain a very potent ATPase activity which also hydrolyses GTP [5]. In order to identify nucleotide binding proteins in membranes which is a necessary prerequisite for purification, GTP-analogs carrying an affinity label were prepared.

Hormones and nucleotides activate adenylyl cyclase in a synergistic fashion. In order to understand how hormone receptor interactions are coupled to nucleotide binding and adenylyl cyclase activation GTP derivatives with a fluorescent reporter group might be useful tools. For that purpose a fluorescent GTP analog was synthesized.

## 2. Materials and methods

ATP, GTP Guanylylimidodiphosphate, Guanylylmethylene diphosphonate, creatinephosphate, creatine kinase were obtained from Boehringer, DL-isoproterenol from Serva (Heidelberg) and DL-propranolol from Sigma. The chemical syntheses of $P^2$-fluoro $P^1$-5′-guanosine diphosphate [GDP(βF)][2], $P^3$-fluoro $P^1$-5′-guanosine triphosphate [GTP(γF)][2], $P^3$-methyl $P^1$-5′-guanosine triphosphate [GTP(γMe)], $P^3$-ethyl $P^1$-5′-guanosine triphosphate [GTP(γEt)], $P^3$-propyl $P^1$-5′-guanosine triphosphate [GTP(γPr)], $P^3$-butyl $P^1$-5′-guanosine triphosphate [GTP(γBu)], $P^3$-phenyl $P^1$-5′-guanosine triphosphate [GTP(γPh)] have been described by Eckstein et al. [7].

The procedures for the preparation of $P^3$-(4-azidoanilido) $P^1$-5′-guanosine triphosphate [GTP(γNH Ph $N_3$)], $N^2$-(4-azidobenzoyl) guanylylimidodiphosphate [2-($NH_3$PhCO)Gpp(NH)p], (Pfeuffer [8]), $P^3$-[*N*(aminoethyl)-1-naphtylamine-8-sulfonate]-$P^1$-5′-Guanosine triphosphate [GTP(γEDANS)], 6-thio-guanylylimidodiphosphate ($^6$S-Gpp(NH)p, *S*-carboxyamidomethylene-6-thio-guanylylimidodiphosphate. (CM-$^6$S-Gpp(NH)p (Pfeuffer and Thomas [9]) will be published elsewhere.

[α-$^{32}$P] ATP (0.5 to 2 Ci per mmol) [8-$^3$H]-Gpp(NH)p (11 Ci per mmole) [8-$^3$H]Gpp($CH_2$)p

*Abbreviations used:* Gpp(NH)p, Guanylylimidodiphosphate; Gpp($CH_2$)p, Guanylylmethylenediphosphonate; GTPγS, Guanosine-5′-*O*-(3-thiotriphosphate).

*The splitting constant $J_{P-F}$ of GDP(βF) and GTP(γF) is not 450 Hz as erroneously reported [7] but 900 Hz.



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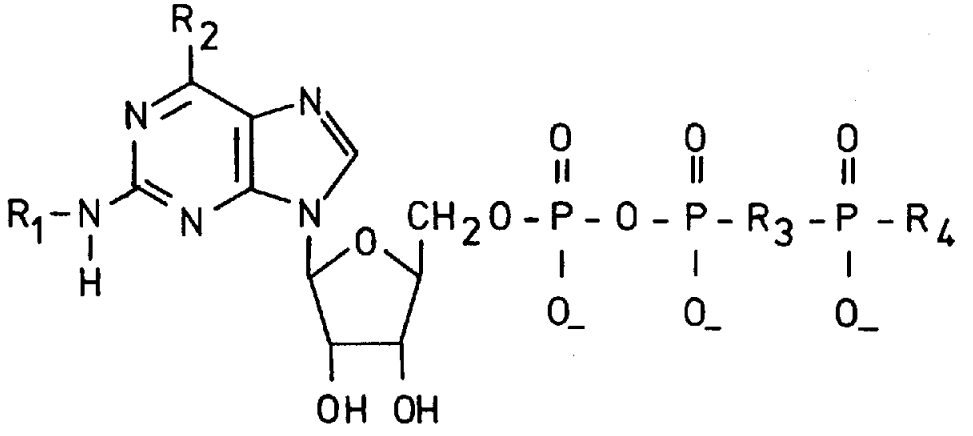
(3–6 Ci per mmol) were purchased from Radiochemical Centre, Amersham, England.

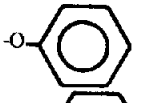
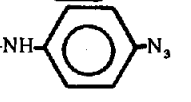
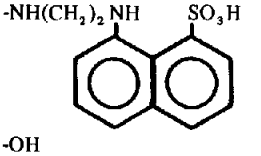
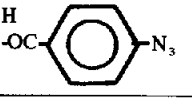
The purity of nucleotides was determined by thin-layer chromatography on polyethyleneimine cellulose plates from Merck, Darmstadt.

Pigeon erythrocyte membranes were prepared according to Øye and Sutherland with the minor modifications described in [4].

Adenylyl cyclase was solubilized as described in [5]. Binding of radioactive nucleotides was studied according to Pfeuffer and Helmreich [5]. The ability of various nucleotide analogs to displace com-

Table 1
Structural formula of GTP analogs



| | | -$R_1$ | -$R_2$ | -$R_3$ | -$R_4$ |
|---|---|---|---|---|---|
| I | GTP | H | -OH | -O- | -OH |
| II | GTP (γF) | H | -OH | -O- | -F |
| III | GTP (γMe) | H | -OH | -O- | $-OCH_3$ |
| IV | GTP (γEt) | H | -OH | -O- | $-OC_2H_5$ |
| V | GTP (γPr.) | H | -OH | -O- | $-OC_3H_7$ |
| VI | GTP (γBu) | H | -OH | -O- | $-OC_4H_9$ |
| VII | GTP (γPh) | H | -OH | -O- |  |
| VIII | GTP (γNHPh$N_3$) | H | -OH | -O- |  |
| IX | GTP (γEDANS) | H | -OH | -O- |  |
| X | Gpp(NH)p | H | -OH | -NH- | -OH |
| XI | $S^6$Gpp(NH)p | H | -SH | -NH- | -OH |
| XII | CM-$S^6$Gpp(NH)p | H | $-SCH_2CONH_2$ | -NH- | -OH |
| XIII | $N^2$-($N_3$PhCO)Gpp(NH)p |  | -OH | -NH- | -OH |

petitively Gpp($CH_2$)p from membranes was measured by determining the amount of bound [8-$^3$H]Gpp ($CH_2$)p which was retained on nitrocellulose filters (SM 11306, 0,45, Sartorius, Göttingen). Protein was determined by Lowry's et al. procedure using bovine serum albumin as reference protein [10].

## 3. Results

### 3.1. *Modification in the 6- and $N^2$-position of the guanine ring*

A fluorescent or proteinreactive group could be attached to the guanine moiety by replacing the oxygen in the 6-position by a thiogroup. The –C=S group is in tautomeric equilibrium with the –C–SH form. 6-Thio-Gpp(NH)p (XI, table 1) activates adenylyl cyclase in pigeon erythrocyte membranes compared to Gpp(NH)p about one half as much. The thio-analog still binds strongly to the nucleotide binding site: $K_i$ was $10^{-6}$ M determined by competition with Gpp($CH_2$)p (see table 2). Carboxamidomethylation of the 6-thio group with 2-iodoactamide decreased considerably activation and affinity (XII) (table 1). This might be due to steric hindrance and/or might be explained by assuming that the GTP binding site only recognizes the keto- but not the enolic form of the guanine part of GTP.

The 2-$NH_2$ group of GMP can be selectively acylated because the 2′ and 3′-acyl-groups of the ribose are much more labile towards dilute alkali than the 2-NH-acyl-group [11]. $N^2$-(4-azidobenzoyl) Gpp(NH)p (table 1) (XIII) however had too low an affinity ($K_i$ = 12.5 μM) to be useful as affinity label.

### 3.2. *Modification of the terminal phosphate group of GTP*

Removal of one negative charge of the terminal phosphate group of GTP does neither abolish binding or adenylcyclase activation. This is different from other nucleosidetriphosphate dependent enzymes, such as myosin ATPase [12] and again indicates that an energy rich phosphate bond is not essential for

Table 2
Binding of GTP analogs to pigeon erythrocyte membranes and activation of adenylyl cyclase

| Nucleotide added | ($K_{diss}$) for binding (μM) | Activation of adenylyl cyclase (pmoles × mg$^{-1}$ × min$^{-1}$) |
|---|---|---|
| None | – | 45 |
| GTP | –[a] | 50–75[b] |
| Gpp(NH)p | 0.09[c] | 430 |
| $^6$S-Gpp(NH)p | 1.0[d] | 190 |
| CM-$^6$S-Gpp(NH)p | 15.0[d] | 70 |
| 2-($N_3$PhCO)Gpp(NH)p | 12.5[d] | 105 |
| GTP (γNHPh$N_3$) | 0.33[e] | 230 |
| GTP (γEDANS) | 0.28[d] | 240 |

Adenylyl cyclase activity was determined in the presence of $10^{-4}$ M of analog and 10 μM DL-isoproterenol. The temperature was 37°C. Incubation was for 20 minutes and an ATP regenerating system was added to [$^{32}$P]ATP. The conditions were those described by Pfeuffer and Helmreich [5].

[a] A reliable value cannot be given, since GTP is degraded by membranes even in the presence of a regenerating system. In the presence of creatine phosphate and creatine kinase an approximate $K_{diss}$ of 0.1 μM was obtained.
[b] Activation by GTP is variable, sometimes even inhibition is observed.
[c] $K_{diss}$ value determined with [8-$^3$H]Gpp(NH)p
[d] $K_i$-values were obtained by competition with [$^3$H]Gpp($CH_2$)p. The concentrations of the latter were 0.2, 0.4, 0.7, 1.2, 2.7 and 5.2 μM. The analog concentration was about twice the $K_i$-value. $K_i$ values were evaluated plotting [$^3$H]Gpp($CH_2$)p (bound)$^{-1}$ versus [$^3$H]Gpp($CH_2$)p (total)$^{-1}$.
[e] $K_{diss}$ value determined with [γ-$^{32}$P]GTP (γNHPh$N_3$).

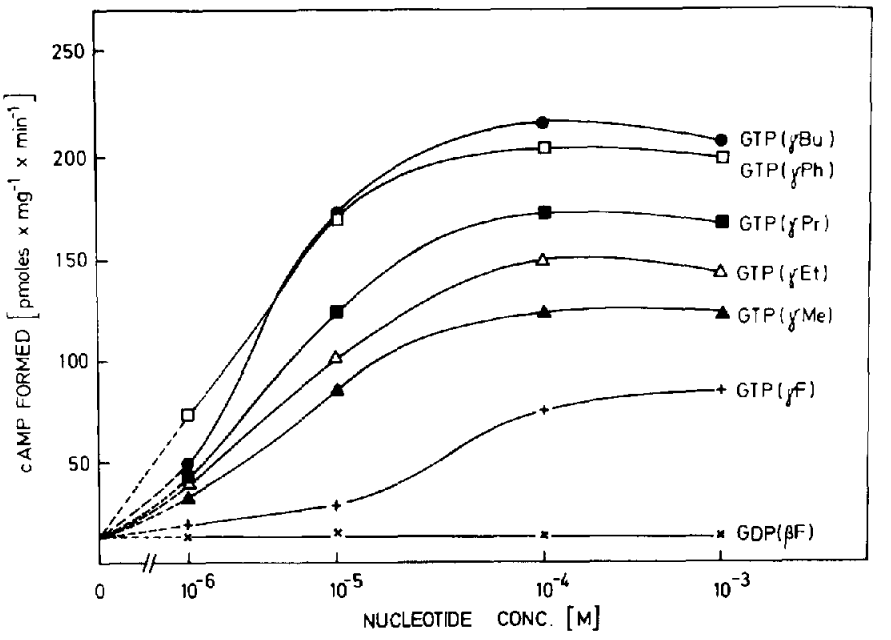



Fig.1. Activation of pigeon erythrocyte membrane adenylyl cyclase by γ-substituted GTP analogs and GDP(βF). Pigeon erythrocyte membranes, 250 μg protein were incubated with GTP analogs in the presence of 10 μM DL-isoproterenol for 20 min at 37°C in a final volume of 100 μl. For adenylyl cyclase assay for 25 min at 37°C 5 μM DL-propranolol were added together with 0.1 mM [α-$^{32}$P]ATP and a regenerating system consisting of 50 μg creatinekinase and 5 mM phosphocreatine [5].

activation [5]. Analogs which have lost one negative charge have lower affinity and are less potent activators of adenylyl cyclase. This applies to compound II in table 1 where the terminal phosphate is replaced by a fluorophosphate. The lower affinity resulting from the loss of one negative charge is amply compensated for by the contribution of hydrophobic binding due to the substituent. However, three phosphate groups seem to be a necessary prerequisite for activation and binding, since GDP(βF) does not activate and binds barely measurable. It only competes with [$^3$H]-Gpp($CH_2$)p at concentrations $> 10^{-4}$ M. From figs.1 and 2 can be seen that activation and affinity increase

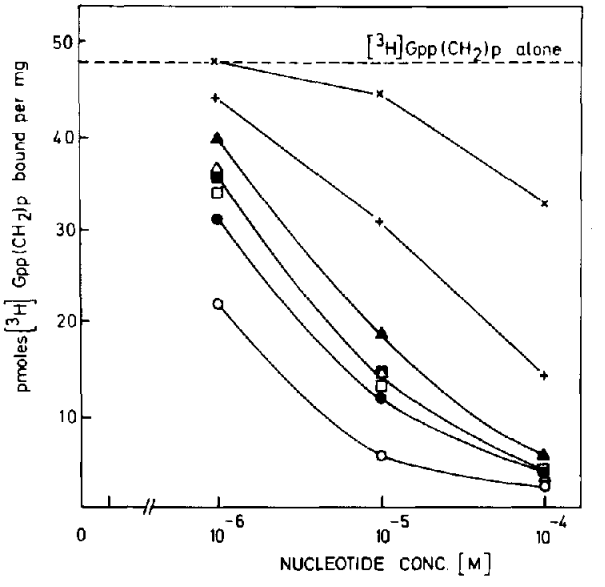



Fig.2. Competitive displacement of [$^3$H]Gpp($CH_2$)p by γ-substituted GTP analogs and GDPβF. Pigeon erythrocyte membranes (210 μg) were incubated in the presence of 2.0 μM [$^3$H]Gpp($CH_2$)p and the GTP analogs of 30 min at 37°C in a total volume of 100 μl. (×——×) GDP (βF); (+——+) GTP (γF); (▲——▲) GTP (γMe); (△——△) GTP (γEt); (■——■) GTP (γPr); (□——□) GTP (γPh); (●——●) GTP (γBu); (○——○) GTP (γNHPh$N_3$).

with growing chainlength of the alkyl substituents of the γ-phosphate of GTP. The greatest activation and affinity is obtained with aromatic esters and amidates of GTP such as GTP (γPh), (table 1, VII) and GTP ($\gamma NHPhN_3$), (table I, VIII). The latter is a potent and useful photoaffinity reagent [8]. This compound binds tight enough to pigeon erythrocyte membranes to allow removal of excess label by repeated washes without loss of activity. This is important for photoaffinity labelling of minor membrane components, constituting only a small fraction of the total membrane protein [8]. γ-Phosphate esters and amidates of GTP are not metabolized in erythrocyte membranes.

The order of affinities, GTP(γF) < GTP(γAlkyl) < GTP(γAryl), for pigeon erythrocyte membrane adenylyl cyclase differs from that found by Eckstein et al. [7] for the elongation factor G-ribosome complex, where GTP(γF) > GTP(γAlkyl) > GTP(γAryl). With the adenylyl cyclase of pigeon erythrocyte membranes, activation and affinity was greatest with a derivative carrying the bulky fluorescent N-aminoethyl-naphthylamine 8-sulfonic acid residue (IX). A similar probe attached to 6-thioinosine via a bromoacetyl spacer was used by Wu and Wu [13] in their studies of RNA polymerase. Using the same coupling procedure and 6-thio-Gpp(NH)p in the case of adenylylcyclase a poorly activating and binding derivative was obtained [14]. Not shown here are experiments with Lubrol PX solubilized adenylylcyclase because the efficacy of the analogs was qualitatively the same.

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