GTP Hydrolysis by Pure Ni, the Inhibitory Regulatory Component of Adenylyl Cyclases*

Teresa Sunyer†, Juan Codina, and Lutz Birnbaumer‡

From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

The stimulatory and inhibitory regulatory components of adenylyl cyclase (N, and Ni), purified to apparent homogeneity without the use of regulatory ligands such as Mg, NaF, and guanyl-5'-yl imidodiphosphate, were tested for GTPase activity by incubating them with [γ-32P]GTP and measuring 32Pi liberation using a charcoal adsorption assay to separate hydrolyzed from nonhydrolyzed radioactivity. We found that Ni is capable of hydrolyzing GTP. The activity was shown to be due to Ni itself and not to presence of one of its minor contaminants by correlating activity with abundance of the 40,000 Da α subunit throughout the last stages of purification and by showing co-migration on a sucrose density gradient of the GTP-hydrolyzing activity with the α, β, and γ subunits of Ni and not with any one of three minor contaminants present in the preparation tested. Preparations of Ni, free of detectable N, exhibited less than 10% of the capacity to hydrolyze GTP, as compared to Ni, on an equal protein basis. The basic properties of the GTP-hydrolyzing activity of Ni were determined. The activity is dependent on Mg ion (apparent K = 5 to 15 mM), and is rapidly lost upon incubation with Mg2+ in the absence of GTP. MgGTP and free GTP serve equally well as substrate (apparent K about 40 mM). Isotopic dilution studies indicate that the GTP-binding site has a relative affinity for guanine nucleotides in the order GTP > GDP > GTPγS > GDPβS > GDPγS with the highest difference (GTP versus GDPβS) being about 10-fold. NaF inhibited GTP hydrolysis by Ni at concentrations at which it activates Ni in intact membranes.

There is ample evidence that Ni, a GTPase. This is based on the following key findings: hormonal stimulation of adenylyl cyclase can often be shown to be associated with increased GTP hydrolysis (1-4); nonhydrolyzable GTP analogs such as GMP-P(NH)P stimulate adenylyl cyclase activity more than GTP (5, 6), a kinetic phenomenon that was mathematically modeled (7, 8); cholera toxin, which covalently ADP-ribosylates the α subunit of N, (9-11) both enhances the effectiveness of GTP (12, 13) and reduces the measurable hormone-dependent increase of GTP hydrolysis, putatively by inhibiting the GTPase activity of N (14); and, finally, reconstitution of pure Ni, which by itself shows very little GTPase activity, with a partially purified (15) or, more importantly, a fully purified β-adrenergic receptor (16), which by itself shows no GTPase activity, results in enhancement of GTP hydrolysis in an agonist-dependent manner.

Indirect kinetic evidence strongly suggests that Ni is likewise a GTPase. Thus, hormone agonism leading to attenuation of adenylyl cyclase activity is associated with enhanced hydrolysis of GTP in several systems (17-19); in S49 cells, despite lack of mitochondrial membranes, devoid of stimulatory regulation by nucleotides, nonhydrolyzable GTP analogs inhibit adenylyl cyclase activity to a more marked degree than GTP (20-22); and, pertussis toxin, which covalently ADP-ribosylates the α subunit of N, (23-27) inhibits both hormonal attenuation of adenylyl cyclase activity (28-30) and inhibitory hormone-mediated stimulation of GTP hydrolysis (19, 31).

It is currently thought that both N and Ni, are regulatory proteins that continuously traverse a kinetic regulatory cycle in which: 1) GTP binds to them; 2) this leads to an "activation" thought to depend, at least in part, on a concomitant subunit dissociation reaction (24, 32, 33); 3) bound GTP is hydrolyzed to GDP with resulting "inactivation" of the regulatory protein which retains GDP bound to it; and 4) release of GDP and/or exchange of GTP for GDP with concomitant reinitiation of the cycle. Many details of the above described kinetic regulatory cycle for N proteins are still unknown. Among these are the points at which reassociation of subunits occurs, the moment at which GDP is released, and, specifically for Ni, whether indeed it is a GTPase. We have recently purified N, to apparent homogeneity from human erythrocytes (27) and have now addressed this last question directly. We found Ni, to be capable of hydrolyzing GTP and report on the characteristics of this activity.

EXPERIMENTAL PROCEDURES

Methods—[γ-32P]GTP was synthesized according to Walsh and Johnson (34) and supplied to us by the Baylor College of Medicine Diabetes and Endocrinology Research Center Center. Activated charcoal was from Sigma (catalogue no. C-5260). It was washed 10 times by sedimentation in distilled water and dried before use. N, was purified as described (27) and kept frozen at -70 °C in a mixture containing 5% Lulobol-PX, 1 mM EDTA, 20 mM β-mercaptoethanol, 150 mM NaCl, 30% ethylene glycol, and 10 mM Na-Hepes, pH 8.0. Nucleotides were from Boehringer Mannheim (GTPγS and GMP-P(NH)P) or Sigma, and were used without further purification. All other reagents were those described recently (27). GTPase Assay—Assays for GTP hydrolyzing capacity of N, were carried out at 32.5 °C in a final volume of 100 μl containing, unless otherwise stated, 500-1000 fmol of N, (50-100 ng of protein), 30 mM...
[\gamma-^{32}P]GTP (about 10^6 cpm/assay), 3 mM MgCl₂, 1 mM EDTA, 0.1% bovine serum, 4 mM \( \beta \)-mercaptoethanol, less than 0.1% Lubrol FX, less than 2 mM NaCl, and 10 mM Na-Hepes, pH 7.5. Incubations were routinely carried out in plastic tubes (27) for 10 min and were stopped by addition of 0.7 ml of ice-cold 5% (w/v) charcoal in 20 mM phosphoric acid (pH 2.1). The mixtures were then allowed to stand on ice for 10-20 min, after which time they were centrifuged for 20 min at 2000 rpm in a clinical centrifuge at 4°C. The \( ^{32}P \), released from \([\gamma-{32}P]GTP \) was then estimated by scintillation counting of 0.5 ml of the clear supernatants mixed with 3.0 ml of ACS scintillation fluid (Amersham). Reaction blanks were between 0.3 and 0.5% of the added \([\gamma-{32}P]GTP \).

Calculation of Free Mg Ion Concentrations (Mg\(^{2+}\))—The concentrations of MgCl₂ (Mg\(_{\text{total}}\)) required to obtain the desired concentrations of Mg\(^{2+}\) under the conditions of EDTA (EDTA\(_{\text{total}}\)) and GTP (GTP\(_{\text{total}}\)) used were calculated according to the equation (55):

\[
M_{\text{tot}} = Mg^{2+}[1 + E_{\text{EDTA}}/K_{\text{EDTA}}] + GTP_{\text{tot}}/[Mg^{2+} + K_{\text{GTP}}]
\]

where \( K_{\text{EDTA}} \) and \( K_{\text{GTP}} \) are the equilibrium dissociated constants of the MgEDTA complex (0.4 \( \mu M \)) and MgGTP complex (60 \( \mu M \)) at pH 7.6. For details, see Ref. 35.

All experiments were repeated between two and four times and representative results are presented on the figures.

All other procedures were recently described (27).

RESULTS

The preparations of N\(_i\) used throughout these studies were those obtained either from step 8 IIb or step 9 I of the purification procedures described recently (27). \( \alpha \) (24, 26), \( \beta \) (24, 26), and \( \gamma \) (33) subunits of N\(_i\), accounted for 90-95% of the Coomassie Blue-stainable material in these preparations which were used without further treatments. Activities observed were therefore due to addition of a form of N\(_i\) that exhibits a molecular weight of 95,000 (27).

As illustrated in Fig. 1, incubation of N\(_i\) with [\( \gamma-^{32}P \)]GTP resulted in release of \( ^{32}P \). This release was proportional to time, the amount of N\(_i\) added and dependent on the concentration of GTP used. GTP hydrolysis was linear for at least 30 min and, as illustrated in Fig. 2, dependent on the presence of free magnesium ion. We investigated the concentrations of GTP and magnesium ion (Mg\(^{2+}\)) required for optimum hydrolytic activity. As illustrated in Figs. 2, 3, and 4, the concentrations of GTP and Mg\(^{2+}\) required to obtain half-maximal activities were rather low. We tested whether the concentration of Mg\(^{2+}\) required was an artifact of the calculations. To this end, we carried out experiments at varying concentrations of Mg\(^{2+}\) in the presence of either 1 mM or 3 mM EDTA. This resulted in the need of adding different concentrations of MgCl₂ to the assays to achieve the same final concentrations of calculated Mg\(^{2+}\). As shown in Fig. 2, the dependence of GTP hydrolysis of Mg\(^{2+}\) was the same at 1 and 3 mM EDTA.

![FIG. 1. TIME COURSES OF GTP HYDROLYSIS BY N\(_i\). Assay conditions were those described on the figure and under "Experimental Procedures." Values are means ± SD of triplicates. A, 100 nM GTP, 2 mM Mg\(^{2+}\); B, 500 fmol of N\(_i\), 2 mM Mg\(^{2+}\).](image)

![FIG. 2. TIME COURSES OF GTP HYDROLYSIS IN THE PRESENCE OF 5 mM EDTA ALONE OR 1 mM EDTA PLUS MgCl\(_2\) TO GIVE 2 AND 5000 \( \mu \)M Mg\(^{2+}\). Mg\(^{2+}\) was calculated and assays were carried out as described under "Experimental Procedures" (500 fmol of N\(_i\), 20 nM GTP). Values are means ± SD of triplicates.](image)

![FIG. 3. EFFECT OF VARYING THE CONCENTRATION OF Mg\(^{2+}\) OR OF ADDITION OF EDTA ON GTP HYDROLYSIS BY N\(_i\). Mg\(^{2+}\) was estimated and assays were as described under "Experimental Procedures" (500 fmol of N\(_i\), 30 nM GTP). Values are means ± SD of triplicates.](image)

An apparent \( K_{\text{act}} \) for Mg\(^{2+}\) of approximately 5 nM was obtained for this preparation of N\(_i\). This value varied somewhat from preparation to preparation ranging from the 5 nM shown here, up to 15 nM. Incubation of N\(_i\) without addition of MgCl₂ and in the presence of 1 mM EDTA still resulted in significant GTP hydrolysis. This hydrolysis could be reduced significantly by including higher concentrations of EDTA (Fig. 3). This suggests that due to the very high affinity of N\(_i\) for divalent cations we appear to have isolated a metal-N\(_i\) complex. Addition of 12 mM MgCl₂ restored hydrolytic activity in the presence of 10 mM EDTA (not shown).

The dependence of hydrolytic activity on GTP concentration (Fig. 4) revealed an apparent \( K_{\text{act}} \) of approximately 40 nM. This value agrees rather closely with the value of 100 nM observed for activation of N\(_i\) in cyc- S49 cell membranes (20, 21). The apparent \( K_{\text{act}} \) of N\(_i\) for GTP was not affected by the concentration of Mg\(^{2+}\) as indicated by the results shown in Fig. 4. Time courses of GTP hydrolysis at 30 nM GTP, such as illustrated in Fig. 2, show them to be essentially the same at 5 mM and 2 \( \mu \)M Mg\(^{2+}\). This is in agreement with the results shown in Fig. 4, indicating that Mg\(^{2+}\) concentration does not affect the apparent \( K_{\text{act}} \) of the system for GTP. Further, since at 5 mM Mg\(^{2+}\), 29.6 nM of 30 nM GTP in the incubation is in the MgGTP form, while at 2 \( \mu \)M Mg\(^{2+}\) only 0.96 nM of the 30 nM GTP is in the MgGTP form, we conclude that N\(_i\) does...
The possibility exists that this conclusion is an over-simplification. We have recently shown that Ni undergoes a complex set of conformational changes and dissociations that depend on MgGTP and guanine nucleotides. This opens the possibility that the apparent lack of effect of a change in abundance of MgGTP or free GTP on hydrolysis of GTP is but a reflection of a rate-limiting step other than the formation of an active enzyme-substrate complex. This thought, suggested to us by a reviewer of this article, has not, however, seriously been explored further.

The data presented thus far allow a calculation of the turnover number of the GTPase of Ni. A value of approximately 0.01 mol hydrolyzed/mol of Ni/min (0.013 for the second approach) was obtained. Overall, the GTP associated with them.

FIG. 4. Effect of GTP concentration on initial rates of GTP hydrolyzed by 500 fmol of Ni. Assays were for 10 min as described under "Experimental Procedures" at either 1 mM MgGTP or 0.8 pmol MgGTP or 0.8 pmol MgGTP. Values are means ± SD of triplicates. Top, GTP hydrolyzed as a function of GTP added; Bottom, Eadie-Hofstee plots of data presented on the top; inset, parameters derived from the Eadie-Hofstee plots.

FIG. 5. Determination of apparent S value of the GTPase activity present in an Ni preparation and correlation with the sedimentation characteristics of Ni. Ni, (7 µg, 70 pmol), 10 µg of albumin, and 10 µg of cytochrome c were subjected to sucrose density gradient centrifugation at 41,000 rpm in the Beckman SW50.1 rotor for 15 h at 4°C as described (27). After completion of the centrifugation the tube was punctured through the bottom and the gradient fractionated in 35 7-drop fractions. One 100-µl aliquot of each fraction was then subjected to sodium dodecyl sulfate-discontinuous urea and polyacrylamide gradient gel electrophoresis (33) followed by Coomassie Blue staining. Another 15-µl aliquot of each fraction was assayed for GTP hydrolytic activity as described under "Experimental Procedures" using 10 nM GTP. A, photograph of the gel stained with Coomassie Blue. The left margin shows position of the molecular weight standards shown in the first lane. The right margin shows position of proteins of interest: I, II, and III, contaminants of Ni; Alb, albumin; α, β, and γ, subunits of Ni; Cyt. c (a), contaminant of cytochrome c preparation used; cyt. c (b), cytochrome c; R, correlation of the relative abundance of α, β, and γ in the fractions of the sucrose density gradient as derived from densitometric scans of the stained gel shown in A with the GTP-hydrolyzing activity in the same fractions. For further details see Ref. 27 and 33.
procedure GTP hydrolysis correlated with abundance of the 40,000 Da αβ subunit regardless of contaminating αδγ or βγ of the 40 K protein (not shown). From preparation to preparation, however, the “specific activity” (i.e., calculated turnover number) varied up to 2-fold. Taken together, these results indicate that GTP hydrolysis is an activity associated with N, and not with one of its nonspecific contaminants. Specific tests for presence of GTP-hydrolyzing activity in N show that, while such an activity is detectable, it is only about 10% as much as that found in N preparations (not shown). Further, since, as mentioned above, GTPase activity of N is essentially the same before and after its separation from N, or the 40,000 Da βγ complex, we conclude that these molecules do not interfere with the GTPase activity of N.

Fig. 6 shows that NaF, but not NaCl, regardless of co-addition of AlCl₃ (38), inhibited GTPase activity of N, with an apparent Kᵢ of approximately 5 mM.

Interaction of the GTP-hydrolyzing Site with Other Nucleotides—Fig. 7 illustrates the results from an experiment in which N was incubated in the presence of 100,000 cpm of [γ-³²P]GTP (specific activity 4,540 cpm/nmol; final concentration 0.22 nM) with increasing concentrations of unlabeled GTP, GDP, GMP-P(NH)₃, GTPγS, and GDPβS. The assay was carried out in two ways. In the first way the reaction was started by adding N to the nucleotide mixtures and measuring the capacity of unlabeled nucleotides to compete with labeled GTP for binding to the GTP-hydrolyzing site of N. In the second way, N was first incubated in the presence of 5 mM MgCl₂ with increasing concentrations of unlabeled nucleotides, thereby allowing them to bind to the GTP-hydrolyzing site of N, and [γ-³²P]GTP was post-added after a 1-min interaction time. The potencies of nucleotides to compete against GTP were GTP > GTPγS > GDP = GMP-P(NH)₃ > GDPβS with the maximum spread being at most one order of magnitude. It is worth noting that the relative affinities were unaffected by preincubation of N with the nucleotides, indicating that under these conditions none of them had led to formation of an “irreversible” guanine nucleotide-N complex from which the nucleotide did not readily dissociate.

DISCUSSION
The data presented here are the first direct demonstration that N, long suspected to be a GTPase, indeed able to hydrolyze GTP. Since the experiments were carried out with an essentially pure protein and the GTP hydrolyzing activity in various preparations, as well as across a sucrose density gradient, correlated only with abundance of N, in the assay and not with any one of several and varying minor contaminants, the results indicate that the GTP-hydrolyzing capacity is intrinsic to the protein itself and not conditioned on presence of additional factors. In the light of recent findings that co-reconstitution of N, which in contrast to N, shows no measurable GTP hydrolytic activity on its own, with R₁ type receptors, such as partially (15) or totally pure receptors (16) promotes the expression of GTPase activity of N, coupled with previous reports that in intact R₁ and N-containing membranes inhibitory hormones stimulate an N-dependent, pertussis toxin-inhibitable GTPase activity. This strongly suggests that R₁ receptors will, like R₂ receptors, be stimulators of the GTPase activity of N, recorded here.

In agreement with this suggestion and during the time that this manuscript was in preparation, Kanaho et al. (36), reported that a GTPase activity of a mixture of α₁ and βγ, purified from bovine brain, is enhanced by addition of rhodopsin-containing membranes.

Although clearly characterizing many heretofore unknown parameters of N, as a GTP-hydrolyzing protein, the experiments performed up to this point do not speak to the important question of whether the hydrolytic activity is associated with a subunit dissociation-reassociation cycle. In another study (37) we have discovered that more cooling of N subunits dissociated under the influence of Mg⁺⁺ and the nonhydrolyz-

---

**Fig. 6.** Effect of varying the concentration of NaCl, NaF, and NaF in the presence of 10⁻⁸ M AlCl₃ on GTP hydrolysis by N. Assays were as described under “Experimental Procedures” (500 fmol of N, 100 nM GTP, 2 mM Mg⁺⁺). Values are means ± SD of triplicates.

**Fig. 7.** Hydrolysis of labeled GTP in the absence and the presence of increasing concentrations of unlabeled guanine nucleotides. Assays were for 10 min as described under “Experimental Procedures” at 2 mM Mg⁺⁺ with 0.22 nM [γ-³²P]GTP present from time 0 (A) or only after a 1-min preliminary incubation of N with the unlabeled nucleotides under GTP assay conditions (B). Results are expressed as percentage of added cpm of [γ-³²P]GTP hydrolyzed after a 10-min incubation. Values are means of triplicates.
able GTP analog GMP-P(NH)P (plus Mg$^{2+}$) or NaF (plus Mg$^{2+}$) leads to their reassociation even in the presence of the dissociating ligands. Due to this, it is impossible for us to stop the reaction initiated by addition of Mg$^{2+}$ and GTP by cooling, and we have not yet developed a way to “freeze” the state which Ni is in when it is actively hydrolyzing GTP, such as able GTP analog GMP-P(NH)P (plus MP) or NaF (plus MP) to cause subunit dissociation in a reversible manner, the results of studies would be necessary to elucidate the effect of GTP and Mg$^{2+}$ on N, subunit structure. The finding that addition of low concentrations of NaF but not equally low concentrations of NaCl resulted in inhibition of the GTP-hydrolyzing capacity of Ni merits comment in this context. On the one hand, this effect can be interpreted as representing a nonspecific action of NaF unrelated to its known effect to activate Ni (20, 21), say by interfering with Mg$^{2+}$ on Ni subunit structure. On the other hand, it may be related to the known effect of NaF to cause Ni subunits to dissociate (24, 38) and therefore be informative about which form of Ni hydrolyzes the GTP. Biochemical separation of the sub-units without the use of an activating ligand that interferes may suggest that the fluoride-affected GTP binding. On the other hand, it may be related to the known effect of NaF to cause Ni subunits to dissociate (24, 38) and therefore be informative about which form of Ni hydrolyzes the GTP. Biochemical separation of the sub-units without the use of an activating ligand that interferes with GTP action will be required before a clear answer to this question can be obtained.

REFERENCES