GTPase activity associates with the inhibitory GTP-binding regulatory component of adenylate cyclase purified from rat brain

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Received 9 April 1984

The inhibitory regulatory component of adenylate cyclase (N_i) was highly purified from rat brain synaptic membranes. A low K_m GTPase activity was always associated with N_i through the purification, and the recovery of GTPase activity correlated well with that of N_i. Purified N_i was hardly ADP-ribosylated by islet-activating protein (IAP). A heat-labile factor in the fraction of the stimulative regulatory component (N_s) restored ADP-ribosylation and also activated the GTPase about 2-fold. NaF which was reported to interact with N_i markedly reduced GTPase activity. The purified N_i fraction inhibited adenylate cyclase only in the presence of a heat-stable factor found in the partially purified regulatory component. GTPase and inhibitory activities were weak in myelin which contained only a small amount of N_i. These findings support the view that GTPase activity is an intrinsic activity of N_i and some factors are necessary for the function of N_i.

GTPase *Inhibitory regulatory component (N_i)* *ADP-ribosylation* *Adenylate cyclase* *Rat brain* *Islet-activating protein*

1. INTRODUCTION

A variety of hormones and neurotransmitters activate or inhibit adenylate cyclase through their receptors on the cell surface. The stimulative and inhibitory GTP-binding regulatory components termed N_s and N_i, respectively, have been proposed to couple these receptors and the catalytic unit of adenylate cyclase [1]. N_s is a dimer composed of a GTP-binding subunit, the substrate of cholera toxin, and a subunit of M_r 35000 [2]. It has been shown that IAP, a toxin from *Bordetella pertussis*, attenuated the receptor-mediated inhibition of adenylate cyclase probably by the ADP-ribosylation of a membrane protein of M_r 41000 [3,4]. This 41-kDa protein, considered to be N_i, was purified as a complex with a 35-kDa protein [5,6]. The 41-kDa protein proved to be a guanine nucleotide-binding protein [5]. However, inhibition of adenylate cyclase by the isolated N_i has not been demonstrated. Stimulative and inhibitory hormones also enhance GTP hydrolysis in plasma membranes which may occur at the GTP-binding sites of these two regulatory components [7,8]. Cholera toxin [7] and IAP [9–11] were reported to prevent the GTP hydrolysis enhanced by stimulative and inhibitory hormones, respectively. This GTPase has not been isolated from membranes.

In [12], we solubilized a low-K_m GTPase activity from synaptic plasma membranes prepared from rat brain. This GTPase was associated with the partially purified regulatory component of

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Abbreviations: IAP, islet-activating protein; N_s and N_i, the stimulative and inhibitory GTP-binding regulatory components of adenylate cyclase, respectively; Gpp(NH)p, 5′-guanylyl imidodiphosphate

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00145793/84/$3.00 © 1984 Federation of European Biochemical Societies

adenylate cyclase and was suggested to be involved in the regulation of adenylate cyclase activity. The partially purified fraction of the regulatory component contained both N_s and N_i as confirmed here. We therefore investigated which regulatory component was associated with the GTPase by separating N_s and N_i. The GTPase activity was copurified with N_i, which supported our hypothesis that the GTPase activity is an activity of N_i.

2. MATERIALS AND METHODS

[γ-^{32}P]GTP and [^{32}P]NAD were purchased from New England Nuclear. IAP was a generous gift from Dr Michio Ui (Hokkaido University, Sapporo). Heptylamine–Sepharose was prepared as in [13]. Sepharose CL-4B (Pharmacia) was used as a matrix.

2.1. *Membranes*

A synaptosome-rich membrane fraction and myelin were prepared from rat brain as in [14].

2.2. *Solubilization, purification and separation of N_s and N_i*

The synaptosome-rich membrane fraction was homogenized in 0.2 M sucrose, 15 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM Tris–HCl, pH 8.0 (final concentration 27–30 mg protein/ml). The homogenate was mixed with 0.5 vol. of 84 mM sodium cholate, stirred for 1 h, and centrifuged at 105000 × g for 1 h. The supernatant was diluted with 20 mM Tris–HCl (pH 8.0) to reduce the cholate concentration to 21 mM for chromatography. Further purification by DEAE–Sephacel, Ultrogel AcA 34 and heptylamine–Sepharose chromatography in the presence of cholate was performed as in [2] for the purification of N_s from rabbit liver, except that ATP was replaced by 20 μM $AlCl_3$ in all buffers [15]. Bovine serum albumin (final concentration 1 mg/ml) was added to the fractions eluted from a heptylamine–Sepharose column to stabilize GTPase and the regulatory components. The fractions containing N_i and N_s were diluted with 2–3 vols of 20 mM Tris–HCl (pH 8.0) and concentrated to about 10% of the original volume by ultrafiltration with Amicon PM-10 and PM-30 membranes, respectively. Lubrol PX (final concentration 0.5%, w/v) was added to the concentrated fractions.

2.3. *Partially purified regulatory component*

In our earlier study on the adenylate cyclase system, we used the partially purified fraction of the regulatory component, which contained N_s and N_i as confirmed here. The properties of this fraction have been studied by us [12] and in [16]. The regulatory component was solubilized from the synaptosome-rich membrane fraction with cholate and ammonium sulfate and partially purified by Sepharose 6B chromatography to remove the catalytic unit as in [16]. The fraction solubilized from myelin was used without purification by gel chromatography as myelin has no catalytic unit [14]. These fractions were stored at −80°C and used in the experiments detailed in tables 3 and 4.

2.4. *Assay of activities*

Before the assay of activities, an aliquot of column fraction (usually 40–50 μl) was diluted with 10 vols of a buffer containing 14 mM cholate, 7 mg/ml phospholipid and 25% saturated ammonium sulfate, and then mixed with 11 vols of saturated ammonium sulfate (pH 7.6). Precipitated protein was dissolved in a buffer containing 7 mg/ml phospholipid and 0.1% (w/v) Lubrol PX [12]. When the amount of protein was low, 10–20 μg/tube of γ-globulin was added as a carrier protein. This procedure was necessary to remove the cholate which interfered with the assay.

GTPase activity was assayed with 2 μM GTP at 37°C for 10 min [12]. Adenylate cyclase activity was reconstituted at 23°C for 3 h from N_s and the partially purified catalytic unit (7.57 μg protein/tube) in the presence of 60 μM Gpp(NH)p [12]. The specific activity of the catalytic unit in the presence of 5 mM $MnCl_2$ was 1620 pmol cyclic $AMP \cdot min^{-1} \cdot mg\ protein^{-1}$. Inhibition of the catalytic unit by the partially purified regulatory component shown in table 4 was measured as in [12] except that 1 μM forskolin was included in the cyclase assay. Adenylate cyclase was assayed for 10 min at 30°C as in [17] except that GTP was omitted. The amount of cyclic AMP formed was determined by the cyclic AMP-binding protein assay [18].

The ADP-ribosylation by IAP (25 μg/ml) was carried out at 25°C for 30 min as in [4] except that

0.1 mM GTP and 5 mM nicotinamide were included in the medium. The reaction was stopped by the addition of 15% (w/v) trichloroacetic acid containing 1% (w/v) SDS. Precipitated protein was washed with 10% (w/v) trichloroacetic acid and then with diethyl ether to remove the residual trichloroacetic acid. Protein was solubilized and subjected to SDS–PAGE as in [19]. The ADP-ribosylated band detected by autoradiography was cut out from the gel and the radioactivity was measured in a scintillation counter.

3. RESULTS AND DISCUSSION

A low-K_m GTPase activity was solubilized with cholate from synaptosome-rich membrane fraction prepared from rat brain. More than 90% of the solubilized GTPase activity was low-K_m activity under the standard assay conditions. The GTPase was further purified by successive chromatography as shown in fig.1. The activity of N_s in the eluted fractions was assayed by the activation of the partially purified catalytic unit in the presence of Gpp(NH)p. The content of N_i was estimated by IAP-catalyzed ADP-ribosylation. GTPase, N_i and N_s activities were eluted in the same fraction from a DEAE–Sephacel column (fig.1a). GTPase activity and N_i were slightly separated from N_s by subsequent Ultrogel AcA 34 chromatography (fig.1b). Peak fractions of GTPase and N_i were collected and then subjected to heptylamine–Sepharose chromatography (fig.1c). GTPase activity was nearly completely separated from N_s by this chromatography, but only a small amount of N_i was recovered in the eluted fractions. Autoradiographs of the protein [^{32}P]ADP-ribosylated by IAP showed that most of the N_i was present in the GTPase fraction (fig.1c). Authors in [20] purified N_i from rabbit liver by a similar procedure although they did not mention GTPase activity and the poor recovery of N_i from a heptylamine–Sepharose column. Our fraction containing N_i and GTPase is probably similar to their fraction of N_i. The fraction containing N_i and GTPase activity is hereafter designated as the 'N_i-GTPase fraction'.

The recovery of GTPase activity in the purification was very different from that of N_s but similar to that of N_i during the first two steps of chromatography (table 1). However, the recovery

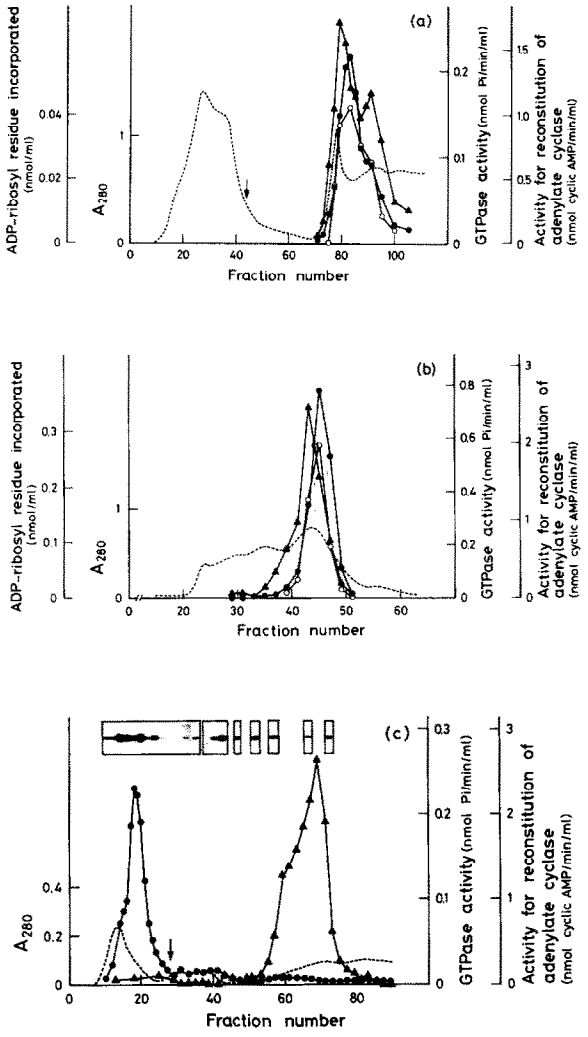

Fig.1. Co-purification of GTPase activity with N_i. The cholate extract (usually 140–155 mg protein) from a synaptosome-rich membrane fraction was subjected to DEAE–Sephacel chromatography (1.6 × 18 cm, 2 ml/fraction) (a). Fractions 77–95 were collected and further purified by Ultrogel AcA 34 chromatography (1.6 × 36 cm, 1.3 ml/fraction) (b). Fractions 40–49 from an Ultrogel column were collected and applied to a heptylamine–Sepharose column (1.0 × 15 cm, 1.2 ml/fraction) (c). The autoradiographs in panel c show the 41-kDa protein that was [^{32}P]ADP-ribosylated by IAP. N_i content (○) was estimated by IAP-catalyzed ADP-ribosylation. N_s activity (▲) was assayed by reconstitution of adenylate cyclase activity with the partially purified catalytic unit. GTPase activity (●) was assayed as described in section 2. (– – –) Absorbance at 280 nm. Elution by a linear gradient was started at the point indicated by the arrow.

Table 1

Comparison of the recoveries of GTPase activity, N_i and N_s

Step	Low-K_m GTPase activity (nmol P_i/min)	ADP-ribosyl residue incorporated (N_i content) (nmol)	Activity for reconstitution of adenylate cyclase (N_s activity) (nmol cyclic AMP/min)
Cholate extract	11.2	5.06	115
DEAE–Sephacel	5.90	2.24	24.9
Ultrogel AcA 34	4.80	2.62	19.4
Heptylamine–Sepharose			
N_i-GTPase fraction	0.85	0.01	not detected
N_s fraction	0.06	trace	31.1

The cholate extract (141 mg protein) from the synaptosome-rich membrane fraction was subjected to chromatography as described in fig.1. The recovery of N_i was estimated by IAP-catalyzed ADP-ribosylation and N_s activity was determined by reconstitution of adenylate cyclase activity with the partially purified catalytic unit. The fraction from a heptylamine–Sepharose column which contained both N_i and GTPase activity was designated as the N_i-GTPase fraction

of N_i from a heptylamine–Sepharose column was quite low compared with that of GTPase activity. We supposed that this apparently poor recovery of N_i could be ascribed to insufficient ADP-ribosylation but not to a true deficiency of N_i in the N_i-GTPase fraction. We found that the activity which markedly enhanced ADP-ribosylation was actually present in the N_s fraction eluted from a heptylamine–Sepharose column. As shown in table 2, efficient ADP-ribosylation in the N_i-GTPase fraction was restored when combined with the concentrated N_s fraction. Thus we could estimate the actual recovery of N_i in the N_i-GTPase fraction as 0.8–1.3 nmol, which was comparable to the recovery of GTPase activity. We also found that GTPase was activated about 2-fold by addition of the N_s fraction (table 2). These activities which enhanced ADP-ribosylation and GTPase activity were heat-labile. The activity to stimulate ADP-ribosylation decreased considerably during storage at −80°C, at which temperature GTPase and N_s activities were fairly stable. Authors in [20] reported that their fraction of N_i similar to our N_i-GTPase fraction lacked the 35-kDa protein usually associated with the 41-kDa protein of N_i. Therefore, the 35-kDa protein may possibly be the necessary factor for ADP-ribosylation and activation of GTPase.

NaF at 10 and 30 mM reduced GTPase activity to 29 and 11% of the control, respectively, as reported for GTPase in synaptic plasma membranes [12]. F^- was reported to induce dissociation of the 41-kDa and 35-kDa proteins of N_i [5]. NaCl at 100 mM slightly enhanced GTPase activity to 112–118% of the control. Trypsin (30 μg/ml) and α-chymotrypsin (300 μg/ml) nearly completely inactivated GTPase. ADP-ribosylation of N_i did not

Table 2

Effects of the N_s fraction on ADP-ribosylation of N_i by IAP and GTPase activity

Fraction	ADP-ribosylation (pmol ADP-ribosyl residue/ml)	GTPase activity (pmol P_i/min per ml)
N_i-GTPase	32	654
N_s	25	100
N_i-GTPase + N_s	510[a]	1390[a]

[a] The activity/ml of the N_i-GTPase fraction is shown

The concentrated N_i-GTPase and N_s fractions from a heptylamine–Sepharose column and the mixture of equal volumes of these fractions were preincubated at 23°C for 30 min. Aliquots were used for ADP-ribosylation and assay of GTPase activity. The N_s activity in the N_s fraction was 14.4 nmol cyclic AMP/min per ml

affect GTPase activity. IAP was reported to prevent inhibitory hormones from enhancing GTPase activity in membranes [9–11], but it is not known whether unstimulated basal GTPase activity related to the function of N_i is also inhibited by IAP.

We previously found that the partially purified fraction of the regulatory component from synaptic plasma membranes inhibited the activity of the catalytic unit of adenylate cyclase [12]. This was confirmed here (see table 4). This inhibitory activity was supposed to be the activity of N_i. Contrary to our expectations, the N_i-GTPase fraction failed to inhibit adenylate cyclase under various conditions. We considered that the failure was due to the lack of a necessary factor which was lost during the purification. To examine this possibility, we employed the partially purified fraction of the regulatory component containing both N_s and N_i (see table 4) as a source of the putative cofactor. The catalytic unit of adenylate cyclase was activated in the presence of excess purified N_s and Gpp(NH)p for the assay of inhibition. The addition of the N_i-GTPase fraction to the activation medium did not inhibit the cyclase. However, the N_i-GTPase fraction added to the partially purified fraction of the regulatory component considerably reduced cyclase activity. Inclusion of the partially purified fraction in the activation medium partly inhibited the cyclase probably due to the N_i present in this fraction. The extent of inhibition by the N_i-GTPase fraction depended on the amount of partially purified fraction added. The partially purified fraction heated in boiling water did not inhibit cyclase activity by itself but still supported inhibition of cyclase by the N_i-GTPase fraction (table 3, exp.2). These results indicate that a certain heat-stable factor, in addition to N_i, is required for the inhibition of adenylate cyclase.

The 35-kDa protein was reported to inhibit or attenuate the activation of N_s by guanine nucleotides and F^- [20]. However, it is unlikely that the inhibition of adenylate cyclase shown in table 3 was induced by the 35-kDa protein released from N_i, since the N_i-GTPase fraction probably lacked the 35-kDa protein. N_i seems to inhibit the cyclase by interacting directly with the catalytic unit. N_i may replace N_s on the catalytic unit or

Table 3

Requirement for a cofactor in the inhibition of adenylate cyclase by N_i-GTPase fraction

Partially purified regulatory component (cofactor fraction) (μg protein)	Adenylate cyclase activity (pmol cyclic AMP/10 min)		Decrease of adenylate cyclase activity by N_i-GTPase fraction (pmol cyclic AMP/10 min)
	− N_i-GTPase	+ N_i-GTPase	
Exp.1			
0	109	99.7	9.3
31	78.2	59.1	19.1
62	71.2	43.3	27.9
Exp.2			
0	77.4	90.3	−12.9
46	54.0	39.1	14.9
46 (heated)	83.0	55.7	27.3

Partially purified catalytic unit (7.57 μg protein) was activated at 23°C for 3 h by purified N_s (18 pmol cyclic AMP/min per tube) and 60 μM Gpp(NH)p. For assay of inhibition, the activation medium included the N_i-GTPase fraction (GTPase activity; 5.45 pmol P_i/min per tube) from a heptylamine–Sepharose column and the indicated amounts of the regulatory component partially purified from a synaptosome-rich membrane fraction as described in section 2. The partially purified fraction of the regulatory component contained a cofactor for the inhibition in addition to N_s and N_i (see also table 4). In exp.2, the partially purified regulatory component was heated for 5 min in boiling water. Adenylate cyclase was assayed at 30°C for 10 min

Table 4

Content of N_i and related activities in myelin

Source of the partially purified regulatory component	Activity for reconstitution of adenylate cyclase (N_s activity) (pmol cyclic AMP/min per mg)	GTPase activity (pmol P_i/min per mg)	ADP-ribosylation by IAP (N_i content) (pmol ADP-ribosyl residue/mg)	Inhibition of adenylate cyclase activity of the catalytic unit (% inhibition)
Myelin	142	11	2.9	6
Synaptic membranes	203	138	32	38

Partially purified regulatory component was prepared from myelin and a synaptosome-rich membrane fraction as described in section 2. N_s activity was determined by reconstitution of adenylate cyclase activity, and N_i content was estimated by IAP-catalyzed ADP-ribosylation. Inhibition of activity of the catalytic unit by the regulatory component (16.2 μg protein/tube) was assayed at 30°C for 10 min in the presence of 1 μM forskolin. The activity of the catalytic unit in the absence of the regulatory component was 105 pmol cyclic AMP/10 min per tube

bind to a different site on the catalytic unit. The latter possibility is more likely because further addition of purified N_s to the activation medium did not greatly prevent inhibition by the N_i-GTPase fraction.

We reported that myelin had very weak GTPase activity despite the N_s activity which was comparable to that in synaptic plasma membranes [12,14]. The weak GTPase activity correlated well with the low content of N_i in myelin (table 4). The activity to inhibit the catalytic unit was found to be weak in the fraction from myelin (table 4). If myelin is assumed to have abundant factors for the activities of N_i, the N_i content in the fraction from myelin is much lower than that in the fraction from synaptic membranes. The very different ratio between N_s and N_i in the two fractions of the regulatory component suggests that N_s and N_i exist independently, rather than forming a bimolecular complex.

The present study strongly indicates that the low-K_m GTPase solubilized from rat brain synaptosome-rich membrane fraction is an integral part of N_i. GTPase may be the 41-kDa protein of N_i itself, or else the 41-kDa protein and other proteins may be required for GTPase activity. We previously found that a low-K_m GTPase activity in synaptic plasma membranes was somewhat inhibited by cholera toxin [12], but we have not found GTPase activity associated with the purified N_s. The GTPase activity considered to be related to N_s may be lost during solubilization.

It is an intriguing question as to whether the isolated 41-kDa protein of N_i indeed inhibits adenylate cyclase. Here, we showed that the N_i-GTPase fraction actually suppressed adenylate cyclase activity with dependence of a certain heat-stable factor. However, the precise mechanism of inhibition and the identification of the heat-stable factor remain to be elucidated.

ACKNOWLEDGEMENTS

We are very grateful to Dr Michio Ui for giving us IAP. This work was partly supported by a Grant-in-Aid (5837008) from the Ministry of Education, Science and Culture of Japan.

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