The stimulatory guanine-nucleotide regulatory unit of adenylate cyclase from bovine cerebral cortex

ADP-ribosylation and purification

Eva J. NEER,* Lisa G. WOLF* and D. Michael GILL†

*Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School, Boston, MA 02115, and

†Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA 02111, U.S.A.

Hormonal stimulation of adenylate cyclase from bovine cerebral cortex is mediated by a guanine-nucleotide regulatory protein (G_s). This protein contains at least three polypeptides: a guanine nucleotide-binding α_s component and a $\beta\gamma$ component, which modulates the function of α_s . The α_s component from many tissues can be ADP-ribosylated with cholera toxin, but has been unusually difficult to modify in brain. We have improved incorporation of ADP-ribose by including isonicotinic acid hydrazide to inhibit the potent NAD glycohydrolase activity of brain. ADP-ribosylation is further improved by addition of detergent to render the substrates accessible and 20 mM-EDTA to chelate metal ions. Although Mg^{2+} is absolutely required for activation of adenylate cyclase by the GTP analogue guanosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppG), it is not obligatory for p[NH]ppG-stimulated ADP-ribosylation by cholera toxin. Under these conditions, the ADP-ribosylation of brain membranes is not enhanced by a cytosolic protein. We find that there are two major sizes of brain α_s , which we have named ' α_{sL} ', with an apparent M_r of 42000–45000, and ' α_{sH} ' with an apparent M_r of 46000–51000 depending on the gel-electrophoretic system used. The α_{sL} and α_{sH} components can incorporate different amounts of ADP-ribose depending on the reaction conditions, so that one or the other may appear to predominate. Thus we show that incomplete ADP-ribosylation by cholera toxin is not a good indication of the relative amounts of α_s units. Functionally, however, both forms of α_s appear to be similar. Both forms associate with the catalytic unit of adenylate cyclase, but neither of them does so preferentially. There is an excess of each of them over the amount associated with catalytic unit. We have now substantially purified G_s from brain by a modification of the method of Sternweis *et al.* [(1981) J. Biol. Chem. 256, 11517–11526] as well as by a new, simplified, procedure. On SDS/polyacrylamide-gel electrophoresis, the purified brain G_s contains both the 45 and 51 kDa α_s polypeptides revealed by ADP-ribosylation and a $\beta\gamma$ component. Activation of purified α_s by guanine nucleotides or fluoride can be reversed by addition of purified $\beta\gamma$ component. The activated form of purified brain G_s has an M_r of 49000 as determined by hydrodynamic measurements, which is consistent with the idea that the active form of brain G_s is the dissociated one.

INTRODUCTION

Cholera toxin catalyses the ADP-ribosylation of G_s , the guanine-nucleotide-binding protein that mediates the stimulation of adenylate cyclase activity by many hormone receptors. The site of ADP-ribosylation is on the α -subunit (α_s), the component of G_s that binds guanine nucleotides. This modification inhibits the GTPase activity associated with G_s and allows GTP to become an effective activator of adenylate cyclase (reviewed by Gill, 1982). The cholera toxin-catalysed reaction is useful not only because it changes the function of adenylate cyclase but also because it permits the attachment of a radioactive label to G_s and thereby

facilitates the study of this protein's properties and behaviour.

We wanted to use the cholera-toxin reaction to help us investigate the adenylate cyclase system of brain tissues. The brain enzyme differs in several respects from adenylate cyclase of non-neural origin. In homogenates, the specific enzymic activity is higher than the activity from non-neural cells (Klainer *et al.*, 1962), it reaches maximal activation by p[NH]ppG extremely slowly (Neer, 1978), it is activated, not inhibited, by non-ionic detergents (Perkins & Moore, 1971; Johnson & Sutherland, 1973), and it is inhibited by EGTA (Bradham, 1972; Neer, 1979). It is not known whether these differences are a consequence of variation in the

Abbreviations used: G_s , the guanine-nucleotide regulatory unit which mediates hormonal stimulation of adenylate cyclase; it is composed of α_s and $\beta\gamma$ components; α_s , the polypeptide component of G_s which can be ADP-ribosylated by cholera toxin and which activates the catalytic unit; heavier (α_{sH}) and lighter (α_{sL}) forms of α_s are found in some cells; β , a polypeptide component of both G_i and G_s with an M_r of 35000–36000; γ , a small protein (5–10 kDa) of unknown function associated with β ; G_i , the guanine-nucleotide regulatory unit which mediates hormonal inhibition of adenylate cyclase; it is composed of α_i and $\beta\gamma$; α_i , the polypeptide component of G_i which can be ADP-ribosylated by pertussis (*Bordetella pertussis*) toxin; p[NH]ppG, guanosine 5'-[$\beta\gamma$ -imino]triphosphate; GTP γ S, guanosine 5'-(3-*O*-thio)triphosphate; INH, isonicotinic acid hydrazide; Buffer A, 50 mM-Tris/HCl (pH 8.0)/75 mM-sucrose/6 mM-MgCl₂/1 mM-dithiothreitol/1 mM-EDTA/soybean and lima-bean trypsin inhibitors (1 mg/l)/3 mM-benzamidinium hydrochloride/10 μ M-AlCl₃/10 mM-NaF/0.9% sodium cholate.

guanine-nucleotide regulatory units or in the catalytic unit.

In addition to these functional differences, the α_s component of brain G_s has been unusually difficult to ADP-ribosylate. In the present paper we have identified and solved some of the particular problems associated with the ADP-ribosylation of brain G_s . Brain contains two forms of α_s with different M_r values which can be preferentially ADP-ribosylated by changing the reaction conditions. We can label brain α_s sufficiently to allow us to study the interaction of both α_s components with the catalytic unit of adenylate cyclase.

We have now purified G_s from brain by a simplification of the method of Sternweis *et al.* (1981) and found it to be composed of α and $\beta\gamma$ components. We have used the highly purified components to demonstrate the inhibitory effect of the $\beta\gamma$ subunit on the ability of α_s to activate the catalytic unit of adenylate cyclase. For these studies we used catalytic unit from bovine caudate nucleus which had been resolved from G_s and G_i (Bender & Neer, 1983).

MATERIALS AND METHODS

Preparation of bovine cortical membranes

A 5 g portion of fresh or frozen (-70°C) bovine cerebral cortex was homogenized in a Dounce homogenizer in 50 ml of 'membrane buffer', which consisted of 0.1 M-Tris/HCl, pH 7.6, 10 mM-MgCl₂, 75 mM-sucrose, 1 mM-dithiothreitol, 1 μg of lima-bean trypsin inhibitor/ml, 1 μg of soybean trypsin inhibitor/ml and 3 mM-benzamidine hydrochloride. The homogenate was centrifuged at 30000 *g* for 15 min. The pellet was homogenized again in 50 ml of membrane buffer and centrifuged as described above. The final pellet, which contained membranes and other particulate material, was taken up in 5 ml of membrane buffer to a protein concentration of 30–50 mg/ml and was stored at -70°C .

ADP-ribosylation of G_s

Brain membranes were first incubated for 10 min at 25°C with all the ingredients of the final incubation except for the NAD. [³²P]NAD was then added and the mixture was incubated at 25°C for a further 30 min. After the incubation, the mixture was diluted with 30 vol. of membrane buffer and the membranes were recovered by centrifugation for 15 min at 21000 *g* (12000 rev./min) in a Sorvall SA600 rotor. The concentrations in the second incubation were: protein, 5–8 mg/ml; Triton X-100, 0.1%; INH, 20 mM; ADP-ribose, 40 mM; p[NH]ppG, 0.5 mM; thymidine, 10 mM; EDTA, 20 mM (when stated); activated cholera toxin, 20 μg /ml; dithiothreitol, 0.25 mM; SDS (used to activate the toxin), 0.025%; and [³²P]NAD, typically 10–20 μM . The membrane preparation was diluted 6-fold into the assay; therefore the components of the homogenization buffer were also present at one-sixth the concentrations given above.

[adenylate-³²P]NAD (ICN, Irvine, CA, U.S.A.) was stored at -70°C in small quantities. The radiochemical purity was determined by t.l.c. on cellulose sheets developed with aq. 50% (v/v) ethanol/0.3 M-(NH₄)₂SO₄, pH 5.0. When used, the NAD had a specific radioactivity of between 14000 and 75000 cpm/pmol.

SDS/polyacrylamide-gel electrophoresis

Analysis of the M_r values of peptides was performed routinely on 9–11% (w/v) acrylamide gels prepared as described by Laemmli (1970). Some experiments (as noted) employed 7–15% polyacrylamide gradient gels. For analysis by Ferguson (1964) plots, gels were made with 7, 9, 11, 13 and 15% acrylamide. The 0.75 mm-thick slab gels were electrophoresed at 30 mA/gel in a water-cooled apparatus (Bio-Rad). They were stained with Coomassie Blue, or with AgNO₃ by the method of Merril *et al.* (1981). The M_r markers were: phosphorylase (94000), bovine serum albumin (66000), fumarase (48000), lactate dehydrogenase (35000), carbonic anhydrase (30000) and soybean trypsin inhibitor (21000).

Preparation of Lubrol extracts containing ADP-ribosyl- G_s

A pellet of toxin-treated membranes was suspended in 4 vol. of membrane buffer and made 2% in Lubrol 12A9. The mixture was stirred on ice for 15 min and centrifuged at 44000 *g* for 45 min. The supernatant fluid was removed and used for further experiments.

Determination of the hydrodynamic properties of G_s

The sedimentation coefficient and Stokes' radius of G_s were determined as described in a previous paper from this laboratory (Neer & Salter, 1981). The precise conditions are given in the legends to the Figures and Tables describing the experiments.

Assay of adenylate cyclase activity

Adenylate cyclase was assayed by a modification of the method of Krishna *et al.* (1968) described by Neer *et al.* (1980). The 70 μl reaction mixture contained 0.5 mM-ATP, 70–90 c.p.m. of [³H]ATP (Amersham)/pmol, 10 mM-MgCl₂, 12 mM-phosphocreatine (Boehringer-Mannheim), creatine kinase (Boehringer-Mannheim, 0.4 mg/ml, 34 units/mg), bovine serum albumin (1 mg/ml), 2 mM-cyclic AMP (Sigma), 0.05–0.1 M-Tris/HCl, pH 7.6, and 50 μM -forskolin (Calbiochem). Radiolabelled ATP was purified before use by passage over a Dowex 50 (X2; H⁺ form) ion-exchange column (Bio-Rad), and eluted with water. Assays of unpurified G_s preparations also contained 3 mM-theophylline (Sigma). The reaction was allowed to proceed for 10 min at 30°C . Assay of G_s activity was carried out by reconstitution with resolved adenylate cyclase catalytic unit from caudate nucleus prepared by the method of Bender & Neer (1983). The catalytic unit was diluted 10–20-fold before the reconstitution in order to decrease the concentration of cholate and (NH₄)₂SO₄. The final concentration of cholate in the assay was 0.25–0.50 mM, and the concentration of (NH₄)₂SO₄ was 20–40 mM. Unless otherwise stated, the routine reconstitution assay contained 20 μM -p[NH]ppG.

Purification of G_s

G_s was purified from bovine brain by two procedures. The first method is a modification of the method of Sternweis *et al.* (1981). The initial steps for purification of G_s are the same for G_i and have been described (Neer *et al.*, 1984). The elution profile of G_s on DEAE-Sephacel II is shown in Fig. 1 of that paper. G_s activity was eluted from DEAE-Sephacel II in two approximately equal peaks. The first, at the start of the NaCl gradient, was

heavily contaminated with many proteins. We were not able to purify G_s from this material. Active fractions in the second peak, which were eluted at a NaCl concentration of about 0.2 M, were pooled and applied to a column of heptylamine-Sepharose synthesized by the method of Shaltiel (1974). The column was equilibrated and eluted as described by Sternweis *et al.* (1981). Most of the protein and a great deal of G_s activity was eluted before the NaCl and cholate gradient. However, approximately one-third of the G_s activity did bind and was eluted with the cholate gradient. This fraction of G_s activity was further purified by a third DEAE-Sephacel column. The 10 ml column was equilibrated with Buffer A and eluted with a 60 ml gradient (from 0 to 0.2 M-NaCl) in Buffer A, with 0.6% Lubrol 12A9 substituted for 0.9% cholate. G_s activity was eluted as a single sharp peak. G_s could be deactivated by passage over hydroxyapatite as described by Sternweis *et al.* (1981), but this led to irreversible loss of 50–80% of the activity.

The second method is a simplification of the procedure devised by Sternweis *et al.* (1981) and used in this laboratory to purify the pertussis-toxin substrates from bovine brain (Neer *et al.*, 1984). We increased the size of the DEAE-Sephacel II column (Table 1 and Neer *et al.*, 1984) to 600 ml and eluted it with an 1800 ml NaCl gradient (from 0 to 0.25 M-NaCl) in a buffer containing 0.05 M-Tris/HCl, pH 8, 6 mM-MgCl₂, 1 mM-dithiothreitol, 1 mM-EDTA, 3 mM-benzamidine, 10 mM-NaF, 10 μ M-AlCl₃ and 0.9% cholate. The second peak of G_s activity was now eluted behind 97% of the protein. This peak was pooled, concentrated 18-fold in an Amicon concentrator, and diluted 11-fold with column buffer, without NaCl but with 0.6% Lubrol 12A9 in place of cholate. The sample was applied to a 30 ml DEAE-Sephacel column equilibrated with the same buffer as described above, but with 0.6% Lubrol 12A9 instead of cholate. It was eluted with a 150 ml gradient containing from 0 to 0.25 M-NaCl. G_s activity was eluted as a single peak at 0.2 M-NaCl.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), as modified by Bailey (1967). Bovine serum albumin was used as the standard. For purified G_s , where the amount of protein was low, protein concentration was determined by automated amino acid analysis (Beckman 121MB amino acid analyser) of an acid hydrolysate of a sample of known volume. The amino acid content was summed to give the total protein concentration in the sample.

RESULTS

Improvements to the ADP-ribosylation conditions in brain membranes

Complete [³²P]ADP-ribosylation of G_s in membranes of tissues other than brain typically requires (1) [³²P]NAD, about 5 μ M, (2) cholera toxin that has been 'activated' with dithiothreitol and SDS to liberate the enzymic A₁ peptide, about 10 μ g/ml, (3) GTP or one of its analogues and (4), in some cases, a cytosolic protein fraction (Gill & Meren, 1978; Gill, 1982). Thymidine (10 mM) is often added to restrict interference from nuclear poly(ADP-ribose) synthesis. The thymidine blocks the appearance of free poly(ADP-ribose), which

Fig. 1. Conditions for ADP-ribosylation of brain α_s .

Effects of adding or omitting individual ingredients. All assays were done with 10 μ M-NAD. Lane 1 shows the result under standard conditions (including EDTA) with no additions or subtractions; lane 2, no cholera toxin; lane 3, the cholera toxin was preincubated only with dithiothreitol; SDS was omitted; lane 4, no INH; lane 5, no Triton X-100; lane 6, no EDTA; lane 7, no p[NH]ppG; lane 8, no thymidine; lane 9, no ADP-ribose; lane 10, the incubation was stopped at zero time.

would otherwise appear on autoradiograms as a 'ladder' of bands corresponding to polymers ranging from decamers to immeasurably large, but it allows a small amount of labelling of the poly(ADP-ribose) polymerase itself (M_r 115000). Although this mixture allows ADP-ribosylation of other membranes, there is little or no toxin-specific ADP-ribosylation of bovine, murine or pigeon brain membrane fractions in this medium. Efficient ADP-ribosylation of brain membranes requires the addition of INH, 0.03–0.1% Triton X-100, 20 mM-EDTA and ADP-ribose. The reasons for including each of these are given below. Fig. 1 shows the consequences of leaving each of these components out of the reaction mixture. INH is a compound reported by Zatman *et al.* (1954) to inhibit the NAD glycohydrolase of birds and ruminants. This is an ectoenzyme that is generally abundant on the surfaces of macrophages and is probably present on the phagocytic cells of brain. It is so active in brain that it prevents effective ADP-ribosylation with low [³²P]NAD. Zatman *et al.* (1954) found that 1 mM-INH inhibited bovine brain NAD glycohydrolase by about 90%. However, we needed to add 20 mM-INH for the peak effect. The NAD glycohydrolase inhibitors nicotinamide and bromoacetylpyridine proved inferior to INH. In addition, ADP-ribose decreased NAD hydrolysis further. However, even in the presence of 20 mM-INH and 40 mM-ADP-ribose, there remains some consumption of NAD, and ADP-ribosylation slows appreciably with time. [Since completing these experiments, we have found that NAD survival can be further improved by the NAD analogue 3-acetylpyridine-adenine dinucleotide (Gill & Woolkalis, 1986); D. M. Gill & J. Coburn, unpublished work.] Because of this residual activity, we avoided high membrane concentrations and low NAD concentrations. The ADP-ribose also decreased background labelling. Thymidine was included to block poly(ADP-ribose) synthesis. 3-Aminobenzamide had no additional effect.

Fig. 2. Effects on ADP-ribosylation of preincubating membranes with nucleotides and either Mg^{2+} or EDTA

The standard preincubation conditions were used for 10 min at 25 °C, except that different nucleotides were added and either 1 mM- $MgCl_2$ (●) or 20 mM-EDTA (○). The membranes were then ADP-ribosylated with [^{32}P]NAD. For the point marked 'x', the preincubation included p[NH]ppG and EDTA, but the membranes were then washed. In this case all the ingredients of the preincubation except for the p[NH]ppG were added back for the ADP-ribosylation step. Abbreviations used: p[NH]ppA, adenosine 5'-[$\beta\gamma$ -imido]triphosphate; ADPR- G_s , ADP-ribosylated G_s .

Brain membrane preparations may contain sealed vesicles which exclude cholera toxin, NAD and p[NH]ppG. Therefore we preincubated membranes with detergents to render all of the vesicles permeable. In addition to the 0.025% SDS included with the toxin, we added 0.03–0.1% Triton X-100. There is very little incorporation if neither detergent is added.

Although Mg^{2+} ions are required for activation of adenylate cyclase by p[NH]ppG, they are not absolutely required for p[NH]ppG stimulation of ADP-ribosylation by cholera toxin (Fig. 2). The guanine nucleotide required by cholera toxin binds a second site ('S') distinct from the site on the substrate, α_s (Gill & Meren, 1983). Binding to the 'S' site has a relatively low requirement for metal ions. ADP-ribosylation proceeds very well in the presence of 20 mM-EDTA, providing that the p[NH]ppG concentration is high. Indeed, the 20 mM-EDTA then substantially increases the ADP-ribosylation of brain G_s (compare lanes 6 and 1 in Fig. 1).

ADP-ribosylation of α_s is increased by preincubation of the membranes with ingredients of the reaction medium including p[NH]ppG for 10 min at 25 °C before adding the NAD. However, preincubation at 37 °C leads to a rapid loss of activity.

By using these modifications it is possible to transfer to brain α_s as much or more ADP-ribose as is possible for other tissues. That these labelled proteins are, indeed, components of G_s is confirmed by studies described below. Similar amounts of ADP-ribose can be transferred to the peptides in membranes from caudate nucleus as to

cortical membranes, and the autoradiographic patterns are similar. The conditions also work for pigeon brain membranes. We have not been able to ADP-ribosylate brain G_s after it has been solubilized.

Guanine nucleotide requirement for ADP-ribosylation

A guanine nucleotide triphosphate is required for the ADP-ribosylation of G_s in all systems that have been examined carefully. Likewise, the ADP-ribosylation of brain membranes requires GTP or p[NH]ppG and discriminates strongly against ATP or adenosine 5'-[$\beta\gamma$ -imido]triphosphate (Fig. 2). The comparison between the various nucleotides is clearest in the presence of EDTA, since the removal of Mg^{2+} prevents transphosphorylation of the natural nucleotides.

Cytosolic factors

We have observed a stimulation of the ADP-ribosylation of brain G_s by crude cytosolic fractions from various tissues, but only in the absence of EDTA. This effect, which is heat stable (60 °C, 10 min) may reflect the removal of an inhibitory metal ion.

Apart from this non-specific effect of cytosol, we have asked whether ADP-ribosylation is stimulated by the specific cytosolic protein ('cytosolic factor') that often enhances the ADP-ribosylation of G_s in membranes of other tissues (Enomoto & Gill, 1980; Enomoto & Asakawa, 1982; Schleifer *et al.*, 1982). Neither crude cytosol, nor the purified factor isolated from bovine testis, accelerates ADP-ribosylation of brain G_s under the conditions used. It may be, however, that potential activation by the cytosolic factor is obliterated by the Triton X-100 present in the incubation medium.

Sizes of the ADP-ribosylated peptides

The major toxin-specific products form a prominent pair of radioactive bands on autoradiograms. Their sizes correspond to those that have been found for the α_s of other tissues. The lighter (faster) band, that we call ' α_{sL} ', migrates exactly with α_s of pigeon erythrocytes, which has been variously estimated to have an M_r of about 42000–45000. Our estimates of its size depend somewhat on the gel system, the markers used for calibration and the method used to plot the relationship between M_r and electrophoretic mobility. Such ambiguities are well known and have been discussed at length by Ugel *et al.* (1971). The heavier band, which we call ' α_{sH} ', is always at least 3 kDa larger than α_{sL} . We obtained M_r values of 45000 and 51000 for α_{sL} and α_{sH} by plotting retardation coefficients (K_R) against M_r values, and values of 44000 and 51000 by plotting $\log M_r$ against R_F using gels of constant acrylamide concentration. On 7–15%-(w/v)-polyacrylamide gradient gels the apparent M_r values were 42000–44000 and 46000–48000 for α_{sL} and α_{sH} respectively. Analysis by the method of Ferguson (1964) of the mobilities in Laemmli (1970) gels containing acrylamide concentrations from 7 to 15% showed that the ADP-ribosylated peptides have the same free electrophoretic mobility (Y_0) as the marker proteins, indicating that the covalent modification does not generate anomalous migration behaviour. Solubilization in Lubrol 12A9 also does not alter their migration rates on SDS-containing gels (Fig. 3a).

The distribution of ADP-ribose between the two bands is variable and can be adjusted experimentally. The α_{sL} band tends to dominate when the total labelling is low,

Fig. 3. Solubilization of ADP-ribosylated G_s and (b) distribution of ADP-ribosylated proteins as a function of the NAD concentration

(a) Lane 1, Lubrol-soluble fraction; lane 2, starting material, total ADP-ribosylated membranes; lane 3, residues of control membranes extracted without Lubrol; lane 4, Lubrol-insoluble membrane residue. (b) The NAD concentration was increased by adding unlabelled NAD to the standard mixture. Consequently the specific activity declined. The total NAD concentrations were: 1, 10 μ M; 2, 30 μ M; 3, 88 μ M; 4, 240 μ M; 5, 780 μ M. The M_r values are marked with respect to a set of standard proteins and refer to gels in (b) only. The bands marked '58' and '31' are not proteins but are artefacts generated by impurities in the radiolabelled NAD. The 115000- M_r band is poly(ADP-ribose) polymerase. The SDS/polyacrylamide gel shown contains a 7–15% gradient of acrylamide.

for any one of a variety of reasons, and may be the only detectable product (see lane 6 in Fig. 1 for an example). However, α_{sH} is more abundant, although it is ADP-ribosylated less readily. Consequently, the α_{sH} band is usually more intense when the labelling is more complete. This is most easily achieved by raising the NAD concentration (Fig. 4). In this regard it is interesting that a 47–48 kDa product was the dominant one when rat brain synaptosomes were ADP-ribosylated at relatively high NAD concentrations (Berthillier *et al.*,

Fig. 4. (a) Sepharose 6B gel filtration of solubilized G_s and (b) autoradiographic analysis of 40 μ l samples of fractions from the column effluent shown in (a)

A 2.7 ml sample of Lubrol 12A9 extract was applied to a 1.5 cm $\times$ 42 cm column of Sepharose 6B equilibrated at 4 $^{\circ}$ C with 0.05 M-Tris/HCl (pH 7.6)/10 mM-MgCl₂/75 mM-sucrose/1 mM-dithiothreitol/0.1% Lubrol 12A9/15 μ M-p[NH]ppG. The flow rate was 10.5 ml/h; the fraction size was 0.7 ml; 15 ml were collected before fractions were taken. Adenylate cyclase (▲) and G_s (●) activities were measured as described in the Materials and methods section. About 85% of the G_s activity was recovered from the column. Samples (50 μ l) were counted for radioactivity (x). All (100%) of the applied radioactivity was recovered. Of this, 49% was protein-bound, the remainder was eluted in the column volume and mainly represented unincorporated NAD. ○, α_{sL} ; △, α_{sH} . (b) Samples (40 μ l) from the fractions indicated were applied to a 10% (w/v) acrylamide gel. The dried gel was used to expose Kodak XAR film. The radioautogram shown scanned with a densitometer to determine the relative amounts of α_{sL} and α_{sH} shown in (a).

1982; Enomoto & Asakawa, 1983). There are, in addition, several minor bands that are ADP-ribosylated by cholera toxin to various extents. The most common of them have apparent M_r values of about 17000, 19000,

Fig. 5. Sucrose-density-gradient analysis of solubilized G_s .

Gradients of 5–20% sucrose in $^1\text{H}_2\text{O}$ or $^2\text{H}_2\text{O}$ contained 0.05 M-Tris/HCl, pH 7.6, 10 mM-MgCl₂, 1 mM-dithiothreitol, 0.1% Lubrol 12A9 and 15 μM -p[NH]ppG. Lubrol-solubilized G_s (0.2 ml) taken from the region marked with a bar in Fig. 4(a) was layered on top of a 4 ml gradient and centrifuged at 59000 rev./min (360000 g) in a Ti SW60 rotor for 6 h ($^1\text{H}_2\text{O}$, a) or 13 h ($^2\text{H}_2\text{O}$, b). The tubes were pierced and fractions were collected from the bottom. The calibrating proteins lactate dehydrogenase [LDH, Stokes' radius 7.35 nm (73.5 Å)], malate dehydrogenase [MDH, 4.35 nm (43.5 Å)] and cytochrome c [CYTO, 1.75 nm (17.5 Å)] were included in the sample. The position of cytochrome c was determined from its A_{420} ; the dehydrogenases were assayed as previously described (Neer, 1974). Samples (8 μl) were assayed for G_s activity by reconstitution with resolved catalytic unit (●). Radioactivity (○) was determined in 50 μl samples.

21000, 25000, 53000, 73000, 135000 and 180000 (Fig. 3b). Some of the smaller ones may be myelin basic proteins. The minor ADP-ribosylation targets are in many ways similar to those reported for rat brain synaptosome preparations. The modification of each of these peptides requires a guanine nucleotide.

Solubilized ADP-ribosylated G_s

After they had been ADP-ribosylated in the presence of p[NH]ppG and Mg^{2+} , bovine cortical membranes were extracted with Lubrol 12A9 as described in the Materials and methods section. Material released from the membranes included both activated adenylate cyclase and free G_s , which could be detected functionally by a reconstitution assay. Fig. 4(a) shows the elution profiles of adenylate cyclase activity and of free G_s activity after gel filtration of the solubilized membranes. The position of the cyclase activity relative to free G_s is the same as we have previously found for material that had not been modified by cholera toxin (Neer & Salter, 1981).

The Stokes' radius of solubilized adenylate cyclase activated by ADP-ribosylated G_s , 7 nm (70 Å), is similar to that for p[NH]ppG-activated adenylate cyclase (Neer *et al.*, 1980). In the present studies the enzyme was constantly in the presence of the proteinase inhibitors, which were included in the buffers. d'Alayer *et al.* (1983) found a larger Stokes' radius [9 nm (90 Å)] for their preparation of solubilized adenylate cyclase and attributed the difference between their findings and those

of several other laboratories to the prevention of proteolysis. Although it is always hard to be sure that every proteinase has been inhibited, we do not think that our smaller size estimate is 'artefactually' low. We have found, however, that adenylate cyclase tends to aggregate when analysed in buffers containing less than 0.10% Lubrol 12A9 (same as Lubrol PX), even though the original solubilization was at higher detergent concentration. d'Alayer *et al.* (1983) used low concentrations of Lubrol (0.05% in their column and gradient buffers), which may have induced some aggregation.

As expected, the radioactive peak of ADP-ribosylated protein spanned the peaks of enzymic activity (i.e., the complex between the catalytic unit and G_s) and of free G_s . The radioactive polypeptides eluted from the column are shown in Fig. 4(b). This radioautogram was analysed by scanning densitometry, followed by excision and weighing of the scans, which revealed a constant ratio of radioactivity in α_{SL} to that in α_{SH} across the radioactive peak. Thus neither form of α_s seems to associate preferentially with the catalytic unit of adenylate cyclase. To confirm this conclusion, enzyme from the leading edge of the peak of activity in Fig. 4(a) was passed over Sepharose 6B for a second time. The distribution of the two sizes of G_s was the same as before (not shown).

Physical properties of ADP-ribosylated G_s

To determine the M_r of p[NH]ppG-activated ADP-ribosylated G_s we used G_s that had been separated from adenylate cyclase activity by gel filtration (the region

marked with the bar in Fig. 4a). The conditions of the ADP-ribosylation reaction, which included exposure to p[NH]ppG and Mg^{2+} for 40 min at 25 °C, activated G_s , and we added 20 μM -p[NH]ppG to all subsequent column and gradient buffers in order to maintain this active state. We determined the Stokes' radius of p[NH]ppG-liganded ADP-ribosyl- G_s to be 5.7 ± 0.1 nm [57 ± 1 Å ($n = 3$)] by gel filtration over calibrated columns of Sepharose 6B as described previously (Neer & Salter, 1981). The sedimentation coefficient was determined on 5–20% (w/v) sucrose gradients. The partial specific volume ($\bar{v}$) was determined by comparing the sedimentation rates of ADP-ribosyl- G_s in sucrose gradients formed with 1H_2O and 2H_2O (Neer, 1978). A representative pair of gradients is shown in Fig. 5. The value for $\bar{v}$ was calculated as described by Sadler (1979) for all pairwise combinations of four 1H_2O and four 2H_2O gradients. The mean value $\pm$ S.E.M. was 0.74 ± 0.01 ml/g, which is the same as that obtained by Kaslow *et al.* (1980) for unmodified G_s from human erythrocytes. It is somewhat lower than the value obtained for G_s from S49 lymphoma cells by Howlett & Gilman (1980). The sedimentation coefficient for ADP-ribosylated- G_s is 2.8 ± 0.2 S ($n = 8$). From these data we calculated the M_r of activated ADP-ribosylated G_s to be 68000. This is higher than the M_r expected for α_s alone and also higher than that of purified activated brain G_s (see below). The reason for the discrepancy is not clear, although it is possible that in the impure preparation a small protein may be associated with α_s .

The sample used for the sedimentation also contained a smaller ADP-ribosylated polypeptide, shown in Fig. 4(b). On polyacrylamide gradient gels this protein has an apparent M_r of 21000 (Fig. 3b), whereas on 11%-acrylamide gels it has an apparent M_r of 26000–27000. The reason for the discrepancy is not known. Radioautography of SDS/polyacrylamide gels of gradient fractions shows that the radioactive peak near the top of the gradient in Fig. 5 is composed of this peptide. It appears to be a fairly hydrophobic molecule, since its sedimentation rate relative to marker enzymes shifts considerably on changing from 1H_2O to 2H_2O gradients. We do not know its function.

In brain, ADP-ribosylation does not prevent re-association of α_s with $\beta\gamma$. We have previously shown that Lubrol-solubilized G_s could be deactivated by gel filtration in buffer containing 1 mM-EDTA and no Mg^{2+} ions (Neer & Salter, 1981). ADP-ribosylated G_s was separated from p[NH]ppG in this way. The apparent sedimentation coefficient of G_s increased from 2.85 to 4.0 ± 0.1 S ($n = 2$). This value is similar to the apparent sedimentation coefficient of 4.7 ± 0.3 S ($n = 4$) which we determined for Lubrol-solubilized unribosylated unactivated G_s . The sedimentation coefficient of unribosylated G_s was determined as described previously (Neer, 1978; Neer & Salter, 1981). Kahn & Gilman (1984) have reported that ADP-ribosylation decreases the affinity of α_s for $\beta\gamma$. However, there seems to be enough endogenous $\beta\gamma$ in the solubilized preparation to allow reassociation of the subunits despite ADP-ribosylation.

Purification of G_s from bovine cerebral cortex

To confirm that the ADP-ribosylated proteins are, indeed, α_s and to extend our studies of components of adenylate cyclase, we first purified brain G_s by a

Fig. 6. SDS/polyacrylamide-gel electrophoresis of purified G_s .

Lane 1, G_s , purified by the Sternweis (1981) procedure, was analysed by SDS/polyacrylamide-gel electrophoresis on 11%-polyacrylamide Laemmli (1970) gels. The gel was stained with $AgNO_3$ (Merrill *et al.*, 1981). Lane 2, G_s purified by the simplified procedure was analysed as described for Lane 1. The gel is stained with Coomassie Blue.

modification of the method of Sternweis *et al.* (1981) as described in the Materials and methods section. The final specific activity of G_s was 1400 nmol of cyclic AMP/min per mg, calculated as described by Sternweis *et al.* (1981) and using resolved catalytic unit from caudate nucleus for reconstitution. In three consecutive preparations the range of specific activity was 985 and 1480 pmol/min per mg. The specific activity was similar to that reported for G_s purified from other sources (Northup *et al.*, 1980; Sternweis *et al.*, 1981; Hanski *et al.*, 1981; Hanski and Gilman, 1982; Codina *et al.*, 1984).

Fig. 6, lane 1, shows a silver-stained SDS/polyacrylamide-gel-electrophoretic pattern of G_s purified by this procedure. Although highly purified, the preparation contains several polypeptide bands, four of which can be identified as belonging to the guanine-nucleotide regulatory components of adenylate cyclase. The protein bands marked α_s correspond to the α_{sH} and α_{sL} bands which can be [^{32}P]ADP-ribosylated by cholera toxin in brain membranes. The α_{sH} band is always diffuse, whether revealed by protein staining in the purified preparation or by radioautography after [^{32}P]ADP-ribosylation of membranes. The elution position of the α_{sH} protein band coincides with G_s activity on the final DEAE-Sepharose column, on subsequent gel filtration over Ultrogel ACA-34 (LKB) or on sucrose-density-gradient centrifugation. An example of the latter is shown in Fig. 8 (below). There was too little α_{sL} for accurate estimation of its distribution.

The purified preparation (Fig. 6, lane 1) contains a

Table 1. Purification of G_s

The procedures are described in the text. Protein was measured by the method of Lowry *et al.* (1951). The specific activity was measured by reconstitution with a standard preparation of catalytic unit. The specific activity calculated at infinite catalytic-unit concentration is 220 nmol of cyclic AMP/min per mg [see Sternweis *et al.* (1981) and text].

Procedure	Protein (mg)	Sp. activity (nmol of cyclic AMP/min per mg)	Total activity (nmol of cyclic AMP/min)	Recovery (%)
Cholate supernatant	4300	1.05	4503	100
DEAE-Sephacel I	2500	0.4	1267	28
Sephacel 6B	1050	0.3	299	7
DEAE-Sephacel II	37	3.8	140	3
DEAE-Sephacel III	1.8	180	320	7

Fig. 7. DEAE-Sephacel chromatography of G_s (DEAE-Sephacel III)

The peak of G_s activity eluted from DEAE-Sephacel II at 0.2 M-NaCl was pooled, concentrated 18-fold in an Amicon concentrator, then diluted 11-fold into column buffer [0.05 M-Tris/HCl (pH 8.0)/6 mM-MgCl₂/75 mM-sucrose/1 mM-EDTA/1 mM-dithiothreitol/3 mM-benzamide/0.6% Lubrol 12A9]. The column was eluted at 4 °C with a gradient that was five times the volume of the column and which went from 0 to 0.25 M-NaCl in column buffer. Fractions (1.5 μ l) were collected at a flow rate of 7.5 ml/h. Samples (40 μ l) from the fractions were analysed by SDS/polyacrylamide-gel electrophoresis and stained with Coomassie Blue. The distribution of the proteins was quantified by densitometry and the area under each peak was integrated with a Hewlett-Packard recording integrator. The areas are reported in arbitrary units: ●, 51 kDa protein, α_{sH} ; ■, 45 kDa protein, α_{sL} ; ◇, 39 kDa protein, α_o ; △, 36 kDa protein, β ; x, a group of $\approx$ 30 kDa proteins which are the major protein contaminants in the starting material. G_s activity (measured by reconstitution with catalytic unit as described in the Materials and Methods section) peaked at fraction 115.

relatively large amount of a 36 kDa protein, which is the β -component. In the gel shown, the 6 kDa γ -component is in the dye front. The band at 41 kDa marked ' α_i ' was identified as one of the brain substrates for ADP-ribosylation by pertussis toxin (Neer *et al.*, 1984). By this criterion it is one of the guanine-nucleotide-binding proteins which mediate hormonal inhibition of adenylate cyclase (Katada & Ui, 1982a,b) or couple receptors to other effectors (Bokoch & Gilman 1984). The highly purified G_s preparation also contains a prominent 64 kDa protein which is a contaminant in every batch of G_s prepared in this way. This protein is a substrate for a brain tyrosine-specific protein kinase related to pp60^{v-src} (Neer & Lok, 1985), but its function is not known.

The results of the new, simplified, purification procedure are given in Table 1 and Fig. 6, lane 2. Although the preparation still contains contaminating proteins, the prominent band at 64 kDa is absent. The specific activity given in Table 1 is that determined with a single standard dilution of resolved catalytic unit. This allows the progress of the purification to be monitored. However, the specific activity of G_s necessarily depends on the concentration of catalytic unit. To compare different preparations, and to compare with the results of other laboratories, the specific activity was calculated as described by Sternweis *et al.* (1981). The initial rate of activation of different amounts of catalytic unit by different amounts of G_s was plotted against the maximal activation at each catalytic unit concentration. The straight line so obtained was extrapolated to determine V_{max} , at infinite catalytic-unit concentration. The specific activity of G_s prepared by the simplified procedure was 2200 nmol of cyclic AMP/min per mg.

The data in Table 1 show that there is an activation of G_s in the final purification step. The reason for this may be found in the distribution of proteins on DEAE-Sephacel III as shown in Fig. 7. A large amount of the β - γ subunit is separated from G_s at this stage. Since the β - γ unit decreases the activity of α_s (see below), the activation we observed may be due to removal of this inhibitor.

Physical properties of purified G_s

The sedimentation coefficient and Stokes' radius of purified brain G_s were determined by gel filtration and ultracentrifugation as described in the legend to Table 2. The fractions from sucrose-density-gradient centrifugation or gel filtration were assayed for G_s activity by

Table 2. M_r of α_s and activity associated with G_s

Purified G_s was applied to 5–20% sucrose density gradients made in 50 mM-Tris/HCl (pH 7.6)/6 mM-MgCl₂/1 mM-EDTA/1 mM-dithiothreitol/0.1% Lubrol 12A9/16 μ M-p[NH]ppG. Identical gradients containing calibrating proteins (galactosidase, lactate dehydrogenase, malate dehydrogenase, cytochrome *c*) were centrifuged at the same time. After 6 h of centrifugation at 59000 rev./min, 4 °C, in a Beckman Ti SW60 rotor, the bottom of the tube was pierced and fractions collected. Cytochrome *c* was located by its A_{418} . Other enzymes were assayed as described by Neer (1974). The distribution of polypeptides in the gradient was determined by SDS/polyacrylamide-gel electrophoresis of the fractions from the gradient. The gel was silver-stained and the relative amounts of each peptide determined by densitometry. The area under the peaks was calculated with a Hewlett-Packard integrator. G_s activity was determined by reconstitution as described by Bender & Neer (1983). The sedimentation coefficients of α_s and of G_s activity were determined by comparison with the position of calibrating proteins of known sedimentation coefficient. The Stokes' radius was determined by gel filtration on calibrated columns of Ultrogel ACA34 (LKB) equilibrated with the same buffer as was used to form the sucrose density gradients, except that the sucrose concentration was 75 mM. The positions of the calibrating proteins with known Stokes' radius, α_s polypeptide and G_s activity were determined as described above. The M_r values were calculated as described previously (Neer *et al.*, 1980). The value for the partial specific volume ($\bar{v}$) was taken to be 0.74 ml/g, the value we determined for ADP-ribosylated α_s .

	Sedimentation coefficient $s_{20,w}$ (S)	Stokes' radius [nm (Å)]	Apparent M_r
α_s polypeptide	2.7 ± 0.1 (3)	4.4 ± 0.1 (44 ± 1) (2)	51000
G_s activity	2.7 ± 0.1 (3)	4.4 ± 0.1 (44 ± 1) (2)	49000

Fig. 8. Sucrose-density-gradient centrifugation of purified G_s

A 200 μ l sample of purified G_s was applied to a 5–20% sucrose density gradient made in H_2O as described in the legend to Table 2. G_s activity was measured by reconstitution with catalytic unit as described in the Materials and methods section ($\bullet$). Recovery of G_s activity was 60%. Fractions from the gradient were analysed by SDS/polyacrylamide-gel electrophoresis. The position of the polypeptides was determined by densitometry of the silver-stained dried gel and integration of area under the peaks. The area is reported in arbitrary units: $\circ$, 51 kDa protein, α_{sH} ; Δ , 36 kDa protein, β . The position of the peaks of the calibrating enzymes is indicated on the Figure: LDH, lactate dehydrogenase; MDH, malate dehydrogenase; CYTO, cytochrome *c*.

reconstitution with the catalytic unit. The distribution of the polypeptides was determined by SDS/polyacrylamide-gel electrophoresis followed by densitometry of the dried, silver-stained, gel and integration of the peak areas. Fig. 8 shows the position of G_s activity and the distribution of the polypeptides on a sucrose density gradient. The M_r values calculated for purified G_s activity and for the α_{sH} polypeptide are indistinguishable. The amount of α_{sL} was too small to determine the position of the peptide with certainty. The M_r of purified G_s indicates that the α_s and $\beta\gamma$ units are dissociated in the presence of p[NH]ppG.

Functional properties of purified G_s

In agreement with the report by Sternweis *et al.* (1981) we found that G_s can be deactivated by passage over hydroxyapatite. However, we found a 50–80% loss of activity, which is greater than the losses previously reported. It appears that unliganded brain G_s may be more labile than G_s from rabbit liver. Nevertheless, the deactivated G_s can be re-activated by incubation with p[NH]ppG (Fig. 9). Activation occurs slowly, as it does with brain crude G_s (Neer & Salter, 1981).

If activation of G_s is due to dissociation of the α_s and $\beta\gamma$ subunits and formation of guanine nucleoside triphosphate-liganded free α_s according to the scheme:

(where *G* is a guanine nucleoside triphosphate), then addition of excess $\beta\gamma$ should prevent the activation. This should occur even in the presence of an activating guanine nucleotide such as p[NH]ppG or GTP γ S. Fig. 10 shows that G_s activity decreases as $\beta\gamma$ concentration increases. Our purification of the brain β subunit has been described (Neer *et al.*, 1984). The effect of $\beta\gamma$ is specific, since heat-denatured $\beta\gamma$ protein does not affect G_s . The $\beta\gamma$ subunit has no effect on catalytic-unit activity in the absence of G_s .

Table 3 shows that dilution of an activating ligand, in this case the fluoride used to stabilize G_s during purification, leads to a slow deactivation of G_s .

Fig. 9. Rate of activation of purified G_s by p[NH]ppG

Purified G_s was deactivated by chromatography on hydroxyapatite as described in the Materials and methods section. The rate of re-activation was measured by incubating the sample [$1 \mu\text{l}$ of de-activated G_s ($0.01 \mu\text{g}$) diluted to $25 \mu\text{l}$ with 50 mM-Tris/HCl ($\text{pH } 7.6$)/ 10 mM-MgCl_2 /1 mM-dithiothreitol/75 mM sucrose] with $16 \mu\text{M-p[NH]ppG}$ at 23°C for the period indicated by the start of the solid bars. The sample of G_s was then added to the catalytic unit and adenylate cyclase activity was measured for 10 min at 30°C . The period of assay incubation is indicated by the length of the bars. The control, shown by broken bars, was incubated without p[NH]ppG. The activity of the catalytic unit alone was $120 \text{ pmol of cyclic AMP/10 min per } \mu\text{g}$ of catalytic unit. The experiment shown was replicated twice.

accompanied by some irreversible denaturation. The rate of deactivation can be enhanced by addition of the $\beta\gamma$ -subunit. Deactivation of α_s by the $\beta\gamma$ -subunit is reversible. Re-addition of Mg^{2+} , Al^{3+} and F^- leads to prompt re-activation of α_s . These results are consistent with the model proposed by Northup *et al.* (1982, 1983).

DISCUSSION

Bovine brain contains two forms of α_s (α_{sH} and α_{sL}) which can be ADP-ribosylated by cholera toxin. The distribution of ADP-ribosylation between the two forms can be varied experimentally. When labelling is low, α_{sL} may be the only detectable product. When labelling is complete, the larger α_{sH} form predominates. A practical consequence of these observations is that it may be misleading to use the extent of ADP-ribosylation as an index of the relative amounts of α_{sL} and α_{sH} in cell membranes unless the conditions of labelling are

Fig. 10. Deactivation of G_s by the $\beta\gamma$ -subunit

Purified G_s was diluted 17-fold with 50 mM-Tris/HCl ($\text{pH } 7.6$)/ 10 mM-MgCl_2 /1 mM-dithiothreitol/75 mM-sucrose containing $20 \mu\text{M-GTP}\gamma\text{S}$. A $35 \mu\text{l}$ portion of diluted G_s was incubated at 23°C for 15 min with the indicated concentration of native $\beta\gamma$ subunits ($\bullet$) or heat-inactivated (100°C , 1 min) subunits ($\circ$). A portion of G_s equivalent to $2 \mu\text{l}$ of undiluted protein was used for each assay and contained $0.01 \mu\text{g}$ of protein. Resolved catalytic unit was then added to each of the samples to measure the activity of G_s . Each assay mixture contained $20 \mu\text{l}$ of a 1:20 dilution of resolved catalytic unit ($1.0 \mu\text{g}$ of catalytic unit) and a final concentration of $10 \mu\text{M-GTP}\gamma\text{S}$. Catalytic-unit activity was also measured in the presence of the indicated concentrations of native and heat-inactivated $\beta\gamma$ subunit without G_s . The $\beta\gamma$ subunit had no effect on the activity of catalytic unit, which was $80 \pm 5 \text{ pmol of cyclic AMP/10 min per } \mu\text{g}$. This has been subtracted from the data shown. The experiment shown is representative of four similar ones.

carefully defined. It is not yet known whether α_{sL} and α_{sH} are functionally the same. We have now shown that both forms associate with the catalytic unit and activate cyclic AMP production. Our ability to label them differentially may help reveal whether there are any important differences.

After ADP-ribosylation and solubilization, G_s can be separated by gel filtration into a free fraction representing about 70% of the total and a remaining 30% associated with the catalytic unit of adenylate cyclase. This distribution is consistent with our previous conclusion that brain G_s is present in excess over catalytic units (Strittmatter & Neer, 1980). Even when activation of the catalytic unit by p[NH]ppG-activated G_s is maximal, free G_s can still be detected. This excess G_s is functional, since it can activate resolved catalytic units.

The M_r (68000) of ADP-ribosylated unpurified G_s suggests that the guanine-nucleotide-activated protein is dissociated. However, the M_r is larger than that of α_{sH} or α_{sL} . Since studies of impure proteins are often complicated by the possibility of interactions with undefined substances in the crude solutions, we purified G_s from bovine brain by a modification of the method of Sternweis *et al.* (1981). The best preparations we

Table 3. Deactivation of G_s by purified β -component is reversible

Purified G_s was diluted 10-fold with 50 mM-Tris/HCl (pH 7.6)/1 mM-dithiothreitol/75 mM-sucrose to lower the concentration of F^- to 1 mM and that of Mg^{2+} to 0.6 mM. Samples of G_s were incubated at 23° with or without 0.5 μ M- β - γ subunit for the times indicated. After the incubation, one set of samples with and without the β - γ subunit was made 10 mM in F^- , 10 μ M in Al^{3+} and 10 mM in Mg^{2+} to re-activate G_s . The Mg^{2+} concentration in the other set was increased to 10 mM so that Mg^{2+} would be constant in all assays. The activity of G_s was measured by reconstitution with catalytic unit as described in the Materials and methods section. The activity of the catalytic unit alone was 77 pmol of cyclic AMP/10 min per μ g. This activity has been subtracted from the results shown, which are expressed as the increment in activity produced by 2 μ l (0.2 μ g) of G_s . The experiment shown is representative of three similar ones.

First incubation	Additions to the assay after the first incubation	Length of first incubation period (min) ...	G _s activity (pmol of cyclic AMP/10 min per 2 μ l of G _s)		
			5	10	15
No added β - γ	Mg^{2+}		72	65	48
0.5 μ M- β - γ	Mg^{2+}		15	10	2
No added β - γ	F^- , Al^{3+} , Mg^{2+}		70	66	60
0.5 μ M- β - γ	F^- , Al^{3+} , Mg^{2+}		66	65	50

obtained were highly purified, but not yet homogeneous. Two protein bands with molecular masses of 51 and 45 kDa corresponded to the ADP-ribosylated proteins. In addition, there was a 36 kDa β -protein as well as a small amount of the α -subunit of the inhibitory guanine-nucleotide-binding protein. The γ -component stains faintly and is not seen on this gel. In the presence of p[NH]ppG, the M_r of purified G_s was 49000, indicating that the protein is fully dissociated. It is not yet known why p[NH]ppG does not fully dissociate the unpurified G_s .

Activation of brain G_s by guanine nucleotides and fluoride seems to follow the scheme proposed by Northup *et al.* (1982). The pure β - γ component can deactivate G_s . The β -subunit itself has no effect on the activity of the catalytic unit which is free of β by immunoblot analysis with anti- β antibodies and of α_i by absence of substrate for ADP-ribosylation by pertussis toxin (R. Huff, J. W. Winslow & E. J. Neer, unpublished work). We had previously shown that the size of adenylate cyclase increases by approx. 50 kDa upon activation by G_s (Neer *et al.*, 1980; Bender *et al.*, 1984). Thus activation of adenylate cyclase seems to be a consequence of association of α_s with the catalytic unit.

G_s , which we have purified from bovine brain, differs in some respects from cholate-solubilized G_s from rabbit liver, human or turkey erythrocytes (Northup *et al.*, 1980; Sternweis *et al.*, 1981; Hanski *et al.*, 1981; Hanski & Gilman, 1982; Codina *et al.*, 1984). The polypeptide distribution of purified brain G_s differs from that of G_s from rabbit liver, turkey or human erythrocytes. Rabbit liver G_s contains two α_s bands: α_{sH} with an M_r of 52000 and α_{sL} with an M_r of 45000. The predominant form seems to be α_{sL} . Erythrocytes from turkeys and humans contain only the α_{sL} form. In contrast, purified G_s from brain contains predominantly the heavier form of α_s . This is consistent with results of labelling of membrane-bound brain G_s with cholera toxin and [32 P]NAD when labelling is maximal. The functional meaning of these differences in polypeptide distribution is not known. Hudson & Johnson (1980) showed by peptide analysis of [32 P]ADP-ribosylated G_s from S49 lymphoma cells that α_{sH} and α_{sL} share tryptic peptides. We have similar data (not shown) for the brain proteins which suggest that

the smaller form of α_s is related to the larger. Our preparation of G_s differed from the others reported because it included proteinase inhibitors in all the buffers. It is possible that this may account for the different distribution. Alternatively, the results may reflect a fundamental heterogeneity of α_s structure from different tissues and species.

We have previously shown that there are two major substrates for ADP-ribosylation by pertussis toxin in bovine brain (Neer *et al.*, 1984). Like the two α_s proteins, they differ in their susceptibility to modification by toxin. Since we can separate the two proteins, we have been able to define other functional differences between them (Huff *et al.*, 1985; Huff & Neer, 1986). The α_{sH} and α_{sL} proteins have not yet been completely separated. It is a reasonable hypothesis, however, that their functions may be subtly different. Testing this hypothesis will be the basis for future studies.

This work was supported by National Institutes of Health (NIH) grant GM34571 and by a grant from the American Cancer Society BC380A to E.J.N. and NIH Grant AI 16928 to D.M.G.

REFERENCES

- Bailey, J. L. (1967) Techniques in Protein Chemistry, p. 340, Elsevier Publishing Co., New York
- Bender, J. L. & Neer, E. J. (1983) J. Biol. Chem. **258**, 2432–2439
- Bender, J. L., Wolf, L. G. & Neer, E. J. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. **17**, 101–109
- Berthillier, G., d'Alayer, J. & Monneron, A. (1982) Biophys. Res. Commun. **109**, 297–304
- Bokoch, G. M. & Gilman, A. G. (1984) Cell **39**, 301–308
- Bradham, L. S. (1972) Biochim. Biophys. Acta **276**, 434–443
- Codina, J., Hildebrandt, J. D., Sekura, R. D., Birnbaumer, M., Bryan, J., Manclark, C. R., Iyengar, R. & Birnbaumer, L. (1984) J. Biol. Chem. **259**, 5871–5886
- d'Alayer, J., Berthillier, G. & Monneron, A. (1983) Biochemistry **22**, 3948–3953
- Enomoto, K. & Asakawa, T. (1982) Biomed. Res. **3**, 122–131
- Enomoto, K. and Asakawa, T. (1983) J. Neurochem. **40**, 434–439
- Enomoto, K. & Gill, D. M. (1980) J. Biol. Chem. **255**, 1252–1258

- Ferguson, K. A. (1964) *Metabolism Clin. Exp.* **13**, 985–1002
- Gill, D. M. (1982) *ADP-Ribosylation Reactions* (Hayaishi, O. & Ueda, K., eds.), pp. 593–621, Academic Press
- Gill, D. M. & Meren, R. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3050–3054
- Gill, D. M. & Meren, R. (1983) *J. Biol. Chem.* **258**, 11908–11914
- Gill, D. M. & Woolkalis, M. (1986) *Methods Enzymol.*, in the press
- Hanski, E. & Gilman, A. G. (1982) *J. Cyclic Nucleotide Res.* **8**, 323–336
- Hanski, E., Sternweis, P. C., Northup, J. K., Dromerick, A. W. & Gilman, A. G. (1981) *J. Biol. Chem.* **256**, 12911–12919
- Hildebrandt, J. D., Codina, J., Risinger, R. & Birnbaumer, L. (1984) *J. Biol. Chem.* **259**, 2039–2042
- Howlett, A. C. & Gilman, A. G. (1980) *J. Biol. Chem.* **255**, 2861–2866
- Hudson, T. H. & Johnson, G. L. (1980) *J. Biol. Chem.* **255**, 7480–7486
- Huff, R. M. & Neer, E. J. (1986) *J. Biol. Chem.* **261**, 1105–1110
- Huff, R. M., Axton, J. M. & Neer, E. J. (1985) *J. Biol. Chem.* **260**, 10864–10871
- Johnson, R. A. & Sutherland, E. W. (1973) *J. Biol. Chem.* **248**, 5114–5121
- Kahn, R. A. & Gilman, A. G. (1984) *J. Biol. Chem.* **259**, 6235–6240
- Kaslow, H. R., Johnson, G. L., Brothers, V. M. & Bourne, H. R. (1980) *J. Biol. Chem.* **255**, 3736–3471
- Katada, T. & Ui, M. (1982a) *J. Biol. Chem.* **257**, 7210–7216
- Katada, T. & Ui, M. (1982b) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3129–3133
- Klainer, L. M., Chi, Y. M., Freidberg, S. L., Rall, T. W. & Sutherland, E. W. (1962) *J. Biol. Chem.* **237**, 1239–1243
- Krishna, G., Weiss, B. & Brodie, B. B. (1968) *J. Pharmacol. Exp. Ther.* **163**, 379–385
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) *Science* **211**, 1437–1458
- Neer, E. J. (1974) *J. Biol. Chem.* **249**, 6527–6531
- Neer, E. J. (1978) *J. Biol. Chem.* **253**, 1498–1502
- Neer, E. J. (1979) *J. Biol. Chem.* **254**, 2089–2096
- Neer, E. J. & Lok, J. M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6025–6029
- Neer, E. J. & Salter, R. S. (1981) *J. Biol. Chem.* **256**, 12102–12107
- Neer, E. J., Echeverria, D. & Knox, S. (1980) *J. Biol. Chem.* **255**, 9782–9789
- Neer, E. J., Lok, J. M. & Wolf, L. G. (1984) *J. Biol. Chem.* **259**, 14222–14229
- Northup, J. K., Sternweis, P. C., Smigel, M. D., Schleifer, L. S., Ross, E. M. & Gilman, A. G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6516–6520
- Northup, J. K., Smigel, M. D. & Gilman, A. G. (1982) *J. Biol. Chem.* **257**, 11416–11423
- Northup, J. K., Smigel, M. D., Sternweis, P. C. & Gilman, A. G. (1983) *J. Biol. Chem.* **258**, 11369–11376
- Perkins, J. P. & Moore, M. M. (1971) *J. Biol. Chem.* **246**, 62–68
- Sadler, J. E. (1979) *J. Biol. Chem.* **254**, 4443
- Schleifer, L. S., Kahn, R. A., Hanski, E., Northup, J. K., Sternweis, P. C. & Gilman, A. G. (1982) *J. Biol. Chem.* **257**, 20–23
- Shaltiel, S. (1974) *Methods Enzymol.* **34**, 126–140
- Sternweis, P. C., Northup, J. K., Smigel, M. D. & Gilman, A. G. (1981) *J. Biol. Chem.* **256**, 11517–11526
- Strittmatter, S. & Neer, E. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6344–6348
- Ugel, A. R., Chrambach, A. & Rodbard, D. (1971) *Anal. Biochem.* **43**, 410–426
- Zatman, L. J., Kaplan, N. O., Colowick, S. P. & Ciotti, M. M. (1954) *J. Biol. Chem.* **209**, 453–483

Received 16 May 1986; accepted 10 September 1986