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Guanine Nucleotide Dependent Formation of a Complex between Choleragen (Cholera Toxin) A Subunit and Bovine Brain ADP-Ribosylation Factor

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ABSTRACT: Cholera toxin activates adenylyl cyclase by catalyzing the ADP-ribosylation of $G_{s\alpha}$, the stimulatory guanine nucleotide binding protein of the cyclase system. This toxin-catalyzed reaction, as well as the ADP-ribosylation of guanidino compounds and auto-ADP-ribosylation of the toxin A_1 protein (CTA_1), is stimulated, in the presence of GTP (or GTP analogue), by 19-21-kDa proteins, termed ADP-ribosylation factors or ARFs. These proteins directly activate CTA_1 in a reaction enhanced by sodium dodecyl sulfate (SDS) or dimyristoylphosphatidylcholine (DMPC)/cholate. To determine whether ARF stimulation of ADP-ribosylation is associated with formation of a toxin-ARF complex, these proteins were incubated with guanine nucleotides and/or detergents and then subjected to gel permeation chromatography. An active ARF-toxin complex was observed in the presence of SDS and GTPγS [guanosine 5′-*O*-(3-thiotriphosphate)] but not GDPβS [guanosine 5′-*O*-(2-thiodiphosphate)]. Only a fraction of the ARF was capable of complex formation. The substrate specificities of complexed and noncomplexed CTA differed; complexed CTA exhibited markedly enhanced auto-ADP-ribosylation. In the presence of GTPγS and DMPC/cholate, an ARF-CTA complex was not detected. A GTPγS-dependent ARF aggregate was observed, however, exhibiting a different substrate specificity from monomeric ARF. These studies support the hypothesis that in the presence of guanine nucleotide and either SDS or DMPC/cholate, ARF and toxin exist as multiple species which exhibit different substrate specificities.

Choleragen activates adenylyl cyclase by catalyzing the ADP-ribosylation of the stimulatory guanine nucleotide binding protein of the cyclase system ($G_{s\alpha}$)[1] (Stryer & Bourne, 1986; Gilman, 1987; Moss & Vaughan, 1988). Toxin-cata-

lyzed ADP-ribosylation is enhanced by a family of ~19–21-kDa guanine nucleotide binding proteins, known as ADP-ribosylation factors or ARFs (Kahn & Gilman, 1984, 1986; Tsai et al., 1987, 1988; Noda et al., 1990). In addition to stimulating the ADP-ribosylation of $G_{s\alpha}$, ARF enhances several other toxin-catalyzed reactions, including the ADP-ribosylation of arginine, other simple guanidino compounds, and proteins unrelated to the cyclase system, the auto-ADP-ribosylation of the choleragen A_1 subunit, and the hydrolysis of NAD to ADP–ribose and nicotinamide (Tsai et al., 1987, 1988; Noda et al., 1990). The ARF proteins appear to interact directly with the catalytic subunit of toxin, in a GTP-dependent reaction promoted by certain detergents and phospholipids (Tsai et al., 1988; Noda et al., 1990; Bobak et al., 1990).

Two soluble ARF proteins and one from the membrane fraction have been purified from bovine brain (Kahn & Gilman, 1986; Tsai et al., 1987, 1988). Studies of toxin activation by these ARFs and guanine nucleotide binding are consistent with the presence of low- and high-affinity binding sites, expression of which is influenced by detergents and phospholipid, i.e., SDS, cholate, and DMPC (Noda et al., 1990; Bobak et al., 1990). The soluble ARFs could activate the toxin catalytic unit under conditions that permitted either high (DMPC plus cholate) or low (SDS) affinity guanine nucleotide binding (Bobak et al., 1990). Significant differences in the auto-ADP-ribosyltransferase and NAD:protein ADP-ribosyltransferase activities were observed depending on the presence of SDS or dimyristoylphosphatidylcholine/cholate (Bobak et al., 1990). In particular, SDS significantly enhanced ARF stimulation of auto-ADP-ribosylation. The present studies were performed to determine whether under conditions associated with selective enhancement of toxin-catalyzed reactions, a physical association between ARF and the toxin catalytic unit could be detected.

Materials and Methods

Materials. Cholera toxin A subunit (CTA) was purchased from List Biologicals, Campbell, CA; bovine serum albumin from Miles Diagnostics, Kankakee, IL; Sephadex G-25, G-75, and G-100 and low molecular weight protein standards from Pharmacia, Piscataway, NJ; GTPγS, GDPβS, and dithiothreitol from Boehringer Mannheim, Indianapolis, IN; [^{32}P]NAD (30 Ci/mol) and nicotinamide [U-^{14}C]adenine dinucleotide (303 Ci/mol) from New England Nuclear, Boston, MA; GTP, GDP, sodium cholate, DMPC, and 4-chloro-1-naphthol from Sigma, St. Louis, MO; SDS from Bio-Rad, Richmond, CA; Ultrogel AcA 44 from IBF, Savage, MD; prestained protein standards from Bethesda Research Laboratories, Gaithersburg, MD.

Preparation of Proteins and anti-ARF Antibodies. G_s was purified from bovine liver membranes as described by Sternweis et al. (1981) followed by chromatography on hydroxylapatite to remove traces of G_i and other contaminating proteins. sARF II was purified from bovine brain cytosol as described by Tsai et al. (1988). Anti-ARF antiserum was obtained from a rabbit immunized with pure sARF II.

Formation of the ARF–CTA Complex and Separation from Monomeric ARF and CTA. CTA (50–100 μg), sARF II (20–50 μg), 30 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 M NaCl, 0.25 M sucrose, and 20 mM Tris-HCl (pH 8.0), with 100 μM guanine nucleotide and/or detergent as indicated (total volume 150–200 μL), were incubated for 45 min at 23 °C and then for 15 min at 30 °C before separation of the ARF–CTA complex from monomeric ARF and CTA. For comparison with elution positions of the ARF–CTA complex and monomeric forms, and to ensure that all fractions from the void volume to the elution position of cytochrome *c* were assayed, the G-75 and G-100 columns were calibrated for each set of experiments using blue dextran (MW 2×10^6) as a marker for the exclusion volume and ovalbumin and cytochrome *c* as molecular weight protein standards. The Ultrogel AcA 44 column was calibrated not only as described above but also with low molecular weight protein standards [phosphorylase *b* (94K), bovine serum albumin (68K), ovalbumin (43K), carbonic anhydrase (30K), trypsin inhibitor (20K), and lactalbumin (14K)] which were added before transfer of the sample to the gel filtration column equilibrated and eluted with 20 mM Tris-HCl (pH 8.0) containing 2 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 M NaCl, 0.25 M sucrose, 1 mM NaN_3, bovine serum albumin (0.1 mg/mL), and guanine nucleotide as present in the sample. Fractions (150–200 μL) were collected and samples (80 μL) analyzed for ADP-ribosyltransferase activity, protein composition, and ARF immunoreactivity.

Analysis of Column Fractions. Samples (80 μL) of column fractions plus 25 mM potassium phosphate (pH 7.5), 10 μM [^{32}P]NAD (2 μCi), 100 μM GTP, 5 mM $MgCl_2$, 3 mM dithiothreitol, and 20 mM thymidine (total volume 100 μL) were incubated for 60 min at 30 °C before addition of 2 mL of cold 7.5% trichloroacetic acid. Precipitated proteins were dissolved in 1% SDS/5% mercaptoethanol and subjected to electrophoresis in 16% polyacrylamide gels (8 × 10 cm) with SDS (Laemmli, 1970). Gels were stained with Coomassie blue before radioautography (Kodak X-OMAT film). ADP-ribosylation of albumin and auto-ADP-ribosylation of CTA_1 are recorded in arbitrary units based on laser densitometry of autoradiograms. NAD:agmatine ADP-ribosyltransferase activity was assayed as described (Moss & Stanley, 1981).

Duplicate gels were used for immunological detection of ARF. Proteins were transferred to nitrocellulose (Towbin et al., 1979). Nitrocellulose blots were incubated with anti-ARF antiserum (1:2000 dilution) and then with goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad) followed by the 4-chloro-1-naphthol (Sigma) substrate solution (Halpern et al., 1987).

Preparation of Monomeric ARF in Bovine Brain Cytosol. Bovine cerebral cortex (20 g) was homogenized in 40 mL of 20 mM Tris-HCL (pH 8.0)/1 mM EDTA/2 mM dithiothreitol/1 mM NaN_3/0.25 M sucrose/0.5 mM benzamidine/0.5 mM phenylmethanesulfonyl fluoride/1 μg/mL soybean trypsin inhibitor and 1 μg/mL lima bean trypsin inhibitor. The homogenate was centrifuged (18000*g*, 30 min). The supernatant was centrifuged (95000*g*, 4 h), and the resultant supernatant was applied to a column (2.5 × 20 cm) of Sephadex G-25 equilibrated and eluted with buffer A [20 mM Tris-HCl (pH 8.0)/1 mM EDTA/2 mM dithiothreitol/1 mM NaN_3/1 mM benzamidine/0.25 M sucrose/5 mM $MgCl_2$/0.1 M NaCl]. Fractions (31 mL) containing proteins were pooled and dialyzed against 4 L of buffer B (buffer A containing 20% glycerol and added sucrose to a concentration of 0.5 M).

The dialyzed supernatant (1.3 mL, 15.6 mg of protein) was incubated with 100 μM GDP, 30 mM dithiothreitol, 5 mM $MgCl_2$, 3 mM DMPC, and 0.2% cholate for 45 min at 23 °C followed by 15 min at 30 °C before application to a column

[1] Abbreviations: ARF, ADP-ribosylation factor; sARF II, soluble form of bovine brain ARF; CT, cholera toxin; CTA, "A" subunit of cholera toxin; CTA_1, A_1 protein of cholera toxin; G_s, stimulatory guanine nucleotide binding protein of adenylyl cyclase; $G_{s\alpha}$, α subunit of G_s; DMPC, dimyristoylphosphatidylcholine; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate).

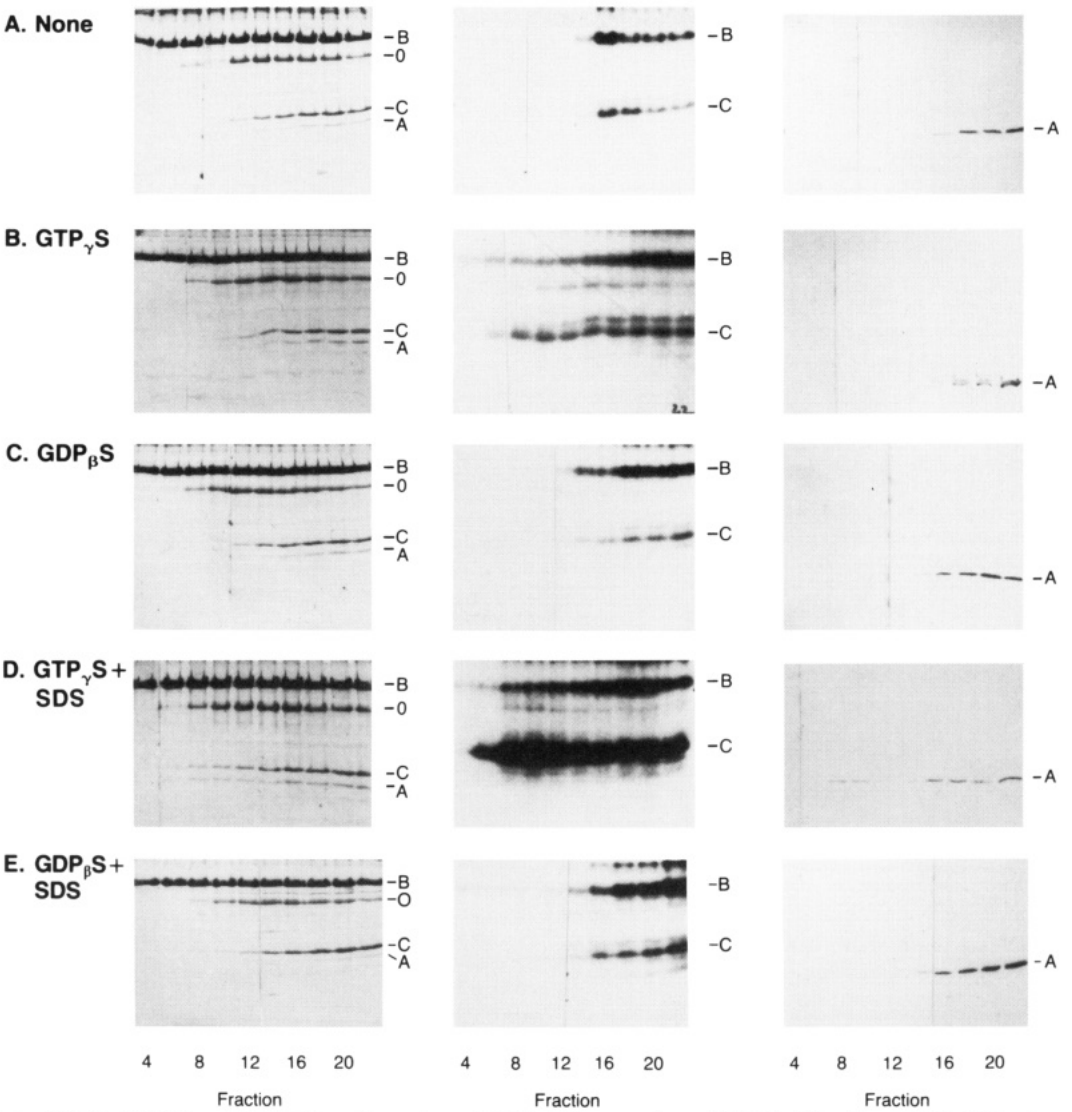

FIGURE 1: Effects of SDS, GTPγS, and GDPβS on formation of ARF–CTA complex. sARF II (25 μg) and CTA (50 μg) were incubated (A) without additions, (B) with 100 μM GTPγS, (C) with 100 μM GDPβS, (D) with GTPγS plus 70 μM SDS, or (E) with GDPβS plus 70 μM SDS. (A–E) Fractions of 4–22. After addition of ovalbumin, mixtures were chromatographed on a column (0.5 × 47 cm) of Sephadex G-75 (Pharmacia). After 3.0 mL of the effluent was discarded, fractions (150 μL) were collected. The tubing connecting the fraction collector to the column had a volume of 1.1 mL, and the void was 3.0 mL. Samples of the indicated fractions were analyzed as described under Materials and Methods. (Left panels) Coomassie blue stained gel. (Middle panels) Radioautograph. (Right panels) Immunoblot with ARF antiserum. (A) ARF; (B) bovine serum albumin; (C) CTA_1 protein ($CT\text{-}A_1$); (O) ovalbumin. The experiment was repeated in its entirety 4 times. As controls in other experiments, part A was reproduced 5 times; part B, 7; part C, 4; part D, 16; and part E, 5.

(1.2 × 44 cm, V_t ~50 mL) of Ultrogel AcA 44 equilibrated and eluted with buffer A containing 100 μM GDP and bovine serum albumin, 0.1 mg/mL. Samples (5 μL) of fractions (1 mL) were incubated with CTA (5 μg) as described for assay of ARF activity. No auto-ADP-ribosylation or ADP-ribosylation of albumin was detected in the early column fractions. With fractions 37–50, which contained apparently monomeric ARF, auto-ADP-ribosylation and ADP-ribosylation of albumin were observed. These fractions were pooled (21 mL) and applied to the Sephadex G-25 column equilibrated and eluted with buffer A containing no sucrose. Fractions containing proteins were pooled (28 mL) and dialyzed for 2 h against 2 L of buffer B followed by concentration (Centricon 10) to ~2 mL (5 mg of protein/mL). This preparation was used in the experiment shown in Figure 6.

RESULTS

After incubation with GTPγS and SDS, but not with GTPγS alone, GDPβS, or no nucleotide, some CTA_1 and ARF, presumably in a high molecular weight complex or aggregate, coeluted near the void volume (fractions 6–10) of a Sephadex G-75 column, as evidenced by protein stain, [^{32}P]ADP-ribosylation, and ARF immunoreactivity (Figure 1A–E). Toxin in these fractions was much more effective in auto-ADP-ribosylation of CTA_1 than was that in fractions 14–36, which contained monomeric CTA and ARF (data not shown). No active complex was formed in the presence of SDS with GDPβS or without nucleotide. After incubation with GTPγS and SDS (without CTA), ARF was apparently entirely monomeric (Figure 2); incubation of CTA with GTPγS and SDS without ARF generated a small amount of inactive aggregated toxin in the stained gels. These data show that formation of active aggregates in the presence of SDS requires both ARF and toxin, in addition to GTPγS.

By use of a relatively long (0.5 × 87 cm) column of Ultrogel AcA 44, the ARF–CTA complex (most in fractions 6–8) was eluted near the void volume ahead of phosphorylase *b* (94 kDa). The complex fractions, which contained roughly equal amounts of visible ARF and CTA_1, auto-ADP-ribosylated CTA_1 but did not effectively ADP-ribosylate albumin (Figure

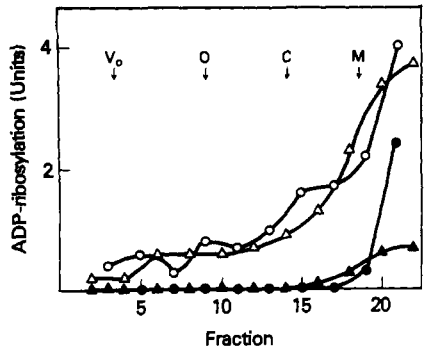

FIGURE 2: Chromatography of ARF or CTA on Sephadex G-100 in the presence of SDS and GTPγS. In the first set of experiments, sARF II (30 μg) was incubated with 70 μM SDS and 100 μM GTPγS (total volume 150 μL). After addition of ovalbumin (100 μg), the mixture was chromatographed on a column (0.5 × 47 cm) of Sephadex G-100. After 3.0 mL of effluent was discarded, fractions (150 μL) were collected. The volume of the connecting tubing was 1.1 mL; the void volume was 3 mL. Samples (80 μL) of the indicated fractions with addition of 1.0 μg of CTA were analyzed as described under Materials and Methods. In a second set of experiments, CTA (50 μg) was incubated with SDS and GTPγS as outlined above, and the reaction mix was chromatographed as noted. ADP-ribosylation of albumin or auto-ADP-ribosylation of CTA_1 in the presence of 1 μg of ARF is recorded in arbitrary units based on laser densitometry of autoradiograms as described under Materials and Methods. V_0, the void volume of Sephadex G-100 column. O, C, or M, elution position of ovalbumin, CTA_1, or monomeric ARF, respectively. (●, O) Incubation containing ARF/SDS/GTPγS; (▲, △) incubation containing CTA/SDS/GTPγS; (△, O) ADP-ribosylation of albumin; (▲, ●) auto-ADP-ribosylation of CTA_1. The experiment was repeated 4 times.

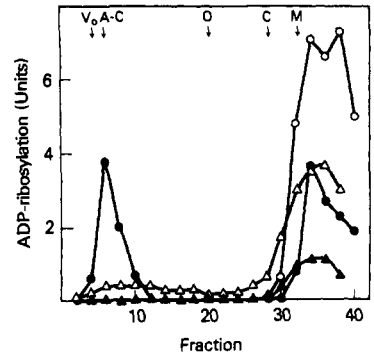

FIGURE 3: Separation of ARF–CTA complex formed in the presence of SDS and GTPγS from monomeric proteins on Ultrogel AcA 44. In experiment I, sARF II (50 μg) and CTA (100 μg) were incubated with 70 μM SDS and 100 μM GTPγS. After addition of standard proteins, the mixture was chromatographed on a column (0.5 × 87 cm) of Ultrogel AcA 44. Fractions (200 μL) were collected after discarding 6 mL of effluent. The exclusion volume was 5.1 mL; the tubing volume was 1.8 mL. Samples of the indicated fractions were analyzed as described under Materials and Methods and in Figure 2. (A–C) Elution position of ARF–CTA complex on the stained gel; the other abbreviations are as noted in Figure 2. (O) ADP-ribosylation of albumin; (●) auto-ADP-ribosylation of CTA_1. The experiment was repeated twice. In the second experiment, fractions 32–41 from experiment I were pooled and concentrated 20-fold. After incubation with 70 μM SDS and 100 μM GTPγS, the mixture was chromatographed on Ultrogel AcA 44 and fractions were analyzed as described in experiment I. (△) ADP-ribosylation of albumin; (▲) auto-ADP-ribosylation of CTA_1.

3). Fractions containing monomeric ARF and CTA, which eluted after the peak of ovalbumin, ADP-ribosylated albumin, but were considerably less active than the complex in auto-ADP-ribosylation of CTA_1 (Figure 3). Monomeric ARF and CTA remaining after formation of the ARF–CTA complex shown in Figure 3 failed to generate active complex on further incubation with SDS and GTPγS. They remained monomeric and were characteristically active in ADP-ribosylating albumin, ARF, and CTA (Figure 3). In this experiment, relatively small amounts of inactive visible CTA_1 and ARF were eluted near the void volume (fractions 6–10); these inactive aggregates may have been formed in part during the concentration step prior to chromatography. In contrast to the previous experiment, formation of an active complex was not observed from the monomeric ARF.

Fractions containing aggregated ARF–CTA complexes or monomeric ARF and CTA were pooled for assay of ADP-ribosyltransferase activity with added G_s and standard proteins (Figure 4). The ARF–CTA complex, with or without added ARF or SDS, auto-ADP-ribosylated CTA_1 but had minimal activity toward other substrates. Addition of ARF plus SDS increased ADP-ribosylation of $G_{s\alpha}$, albumin, and phosphorylase *b* but not auto-ADP-ribosylation of CTA_1. Addition of CTA to the ARF–CTA complex, with or without SDS, increased labeling of $G_{s\alpha}$, albumin, phosphorylase *b*, and CTA_1; there was little or no ADP-ribosylation of ARF under any condition (Figure 4A). In contrast, in assays with unincubated CTA and ARF (without ARF–CTA complex), ADP-ribosylation of ARF was prominent (Figure 4A). It appears that added ARF (in the presence of SDS) can enhance the activity of CTA in the complex fractions toward $G_{s\alpha}$, albumin, and phosphorylase *b* but has little effect on auto-ADP-ribosylation and is itself a poor substrate for this form of the toxin. Conversely, ARF in these fractions can activate, but is not a substrate for, added CTA (with or without SDS). ARF activation of CTA resulted in enhanced ADP-ribosylation of $G_{s\alpha}$, rather than an increase in auto-ADP-ribosylation. The ARF–CTA complex fractions contain ARF and toxin that catalyze the enhanced auto-ADP-ribosylation of CTA_1, as well as ARF that is capable of stimulating the activity of added CTA toward $G_{s\alpha}$, albumin, or phosphorylase *b* and toxin that can be activated by added ARF to ADP-ribosylate these proteins. Thus, there may be different species of ARF and/or toxin in the aggregate; one subset of these species preferentially enhances the ADP-ribosylation of $G_{s\alpha}$, rather than the auto-ADP-ribosylation of CTA_1.

The monomeric pool (Figure 4B) ADP-ribosylated only $G_{s\alpha}$, albumin, and phosphorylase *b*, with some enhancement by added SDS or ARF. ARF plus SDS increased modification of these proteins as well as that of CTA_1 and notably of ARF itself. With added ARF plus SDS, the pattern of ADP-ribosylation was very similar to that seen with CTA, ARF, and SDS that had not been previously incubated and subjected to gel filtration (Figure 4B). Addition of CTA alone to the monomeric pool had very little effect, but in the presence of SDS, it increased labeling of $G_{s\alpha}$, albumin, phosphorylase *b*, CTA_1, and, to a lesser extent, ARF. Thus, although the pool contained CTA in molar excess of ARF and the toxin was susceptible to activation by added ARF, the pool also contained ARF that was capable of activating added CTA. It seems clear that the substrate preferences of the toxin in the ARF–CTA complex and in the monomeric form are different.

After incubation with DMPC/cholate in the presence of GTPγS, a small fraction of ARF appeared visible in the stained gel as an aggregate that was eluted near the void volume of an Ultrogel AcA 44 column (Figure 5). This was much more active in stimulating ADP-ribosylation of serum albumin than was the majority of the ARF, which was apparently monomeric; with DMPC/cholate and GDPβS, no aggregated ARF protein was seen (Figure 5), although there was some ARF activity in fractions 4–10 (Figure 5).

Since only a small fraction of ARF was aggregated in the presence of DMPC/cholate and GTPγS, it seemed important

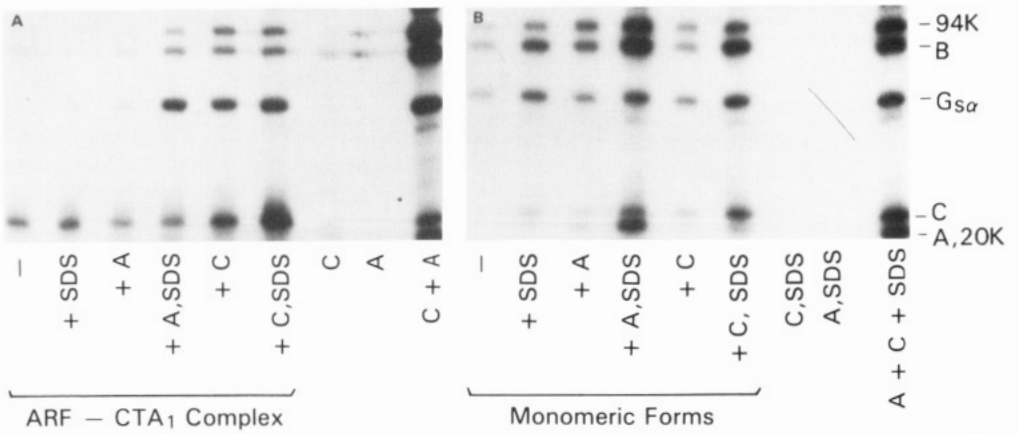

FIGURE 4: ADP-ribosylation of proteins by ARF–CTA complex formed in the presence of SDS and GTPγS and by monomeric proteins. From an experiment like that in Figure 3, fractions 6–10 containing ARF–CTA complex and fractions 32–41 containing monomeric ARF and CTA were pooled separately. Samples (50 μL) of each pool were incubated with G_s (0.2 μg), standard proteins (2 μL), [^{32}P]NAD, 100 μM GTP, etc. as described for ADP-ribosylation of proteins under Materials and Methods. Other additions as indicated were CTA (C, 1 μg), sARF II (A, 1 μg), and 70 μM SDS. After ADP-ribosylation, SDS–PAGE and radioautography were carried out as described under Materials and Methods. For radioautography, Figure 4A was exposed to film 4 times longer than Figure 4B. On the basis of protein staining, amounts of ARF and CTA_1 in the complex pool were roughly equal. In the monomeric pool, there was roughly twice as much CTA as ARF. The experiment was repeated 3 times.

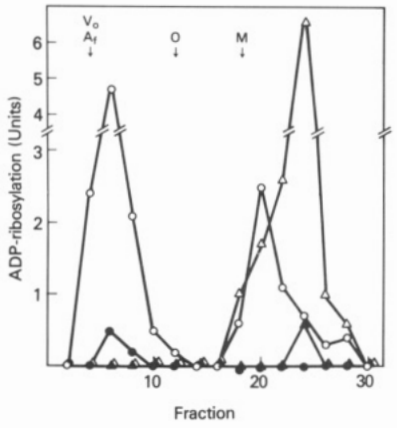

FIGURE 5: Self-association of ARF in the presence of GTPγS and DMPC/cholate. sARF II (30 μg) was incubated with 3 mM DMPC, 0.2% sodium cholate, and 100 μM GTPγS (●, ○) or GDPβS (▲, △). After addition of ovalbumin, the mixture was chromatographed on a column (0.5 × 47 cm) of Ultrogel AcA 44. Fractions (200 μL) were collected after 3.4 mL of effluent was discarded. The void volume was 2.5 mL; the tubing was 1.8 mL. Samples of the indicated fractions plus CTA, 0.5 μg, were analyzed as described under Materials and Methods and in Figure 2. A_f, self-associated ARF; (●, ▲) auto-ADP-ribosylation of CTA_1; (○, △) ADP-ribosylation of albumin. Other abbreviations as in Figure 2. The experiment was repeated 5 times.

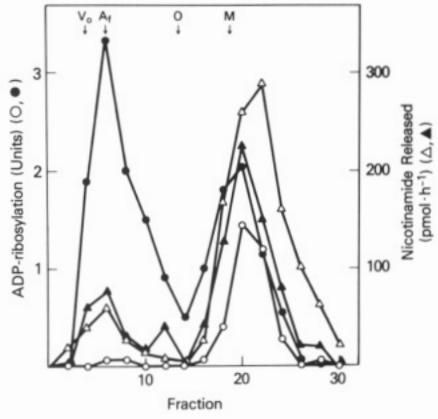

FIGURE 6: Self-association of cytosolic ARF in the presence of GTPγS and DMPC/cholate. The monomeric ARF preparation from brain cytosol (1.25 mg of protein) prepared as described under Materials and Methods was incubated with 3 mM DMPC, 0.2% sodium cholate, and 100 μM GTPγS (●, ▲) or GDPβS (○, △) in a total volume of 250 μL before chromatography on Ultrogel AcA 44 as described in the legend for Figure 5. Samples (80 μL) of the indicated fractions were incubated with CT (5 μg) and other additions as described under Materials and Methods and in Figure 2 to assess ARF enhancement of ADP-ribosylation of bovine serum albumin (●, ○) or with CTA (1 μg) for assay of NAD:agmatine ADP-ribosyltransferase activity (▲, △); in fractions assayed in the absence of CTA, no significant toxin-independent ADP-ribosyltransferase activity was found. Transferase activity is in picomoles of nicotinamide released per hour. A_f, self-associated ARF; the other abbreviations are as noted in Figure 2. The experiment was repeated 3 times.

to determine whether the ability to form aggregates was a result of the purification procedure. A crude preparation of monomeric ARF from brain cytosol was incubated with GDPβS or GTPγS and DMPC/cholate before separation of ARF aggregates from monomers on AcA 44. In the presence of GTPγS but not GDPβS, an aggregated species, much more active than the monomeric ARF in enhancing ADP-ribosylation of albumin, was observed (Figure 6). Although the difference between the two forms was not as marked, the aggregated ARF was also somewhat more effective than the monomeric in stimulating toxin NAD:agmatine ADP-ribosyltransferase activity (Figure 6). As was found with the purified ARF, only a small percentage of the total ARF protein was in the aggregated fractions. These findings are consistent with the conclusion that the existence of ARF aggregates and monomers that exhibit different activities is not a result of the purification procedure.

When purified ARF was incubated with CTA and GTPγS in the presence of DMPC/cholate, a small amount of aggregated ARF, but no CTA_1, was seen on the stained gel (Figure 7). There was, however, ADP-ribosylation of albumin in these fractions consistent with the presence of some toxin (Figure 7). After incubation of ARF and CTA with DMPC/cholate and GDPβS, no aggregated ARF and/or toxin were detected (Figure 7).

Pooled complex fractions from an experiment like that in Figure 7 weakly ADP-ribosylated $G_{s\alpha}$; this was enhanced minimally by ARF or SDS and only slightly by both (Figure 8). Addition of CTA, with or without SDS, greatly increased modification of $G_{s\alpha}$, albumin, and, to a lesser degree, phosphorylase *b* but auto-ADP-ribosylation was minimal (Figure

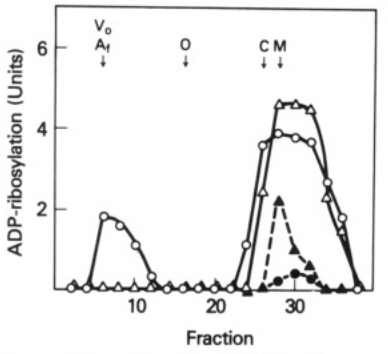

FIGURE 7: Self-association of purified ARF in the presence of CTA, GTPγS, and DMPC/cholate. sARF II (30 μg) and CTA (50 μg) were incubated with 3 mM DMPC, 0.2% sodium cholate, and 100 μM GTPγS (●, ○) or GDPβS (▲, △). After addition of ovalbumin, the mixtures were chromatographed and fractions analyzed as described in Figure 5. A_f, self-associated ARF; the other abbreviations are as noted in Figure 2. (○, △) ADP-ribosylation of albumin; (●, ▲) auto-ADP-ribosylation of CTA_1. The experiment was repeated twice.

8). Thus, these fractions contained very little toxin susceptible to activation by added ARF and relatively much more active ARF. Pooled monomeric fractions ADP-ribosylated $G_{s\alpha}$, albumin, phosphorylase *b*, and, to a small extent, lactalbumin; auto-ADP-ribosylation was minimal but was increased somewhat by SDS. Added ARF (with or without SDS) increased labeling of several proteins and was itself ADP-ribosylated but had very little effect on auto-ADP-ribosylation. The stimulatory effect of ARF and the lack of effect of added CTA are consistent with the fact that CTA was in molar excess of ARF in the monomeric pool.

Discussion

As shown here, GTPγS, but not GDPβS, can alter the physical properties of ARF, enhancing self-association and interaction with CTA to form complexes that can be separated from the apparently monomeric proteins by gel filtration. The protein–protein interactions were markedly influenced by detergents (SDS or DMPC plus cholate). These observations are consistent with the earlier findings that ARF in the presence of GTPγS (but not GDPβS) can activate CTA (Tsai et al., 1987, 1988; Noda et al., 1990). ARF activation, including the K_A for guanine nucleotide, the choice of ADP-ribose acceptor, and the degree of enhancement of toxin activity are influenced in different ways by SDS and DMPC/cholate (Tsai et al., 1988; Noda et al., 1990; Bobak et al., 1990). In the presence of DMPC/cholate, ARF binds GTPγS with high affinity whereas high-affinity binding cannot be demonstrated in the presence of SDS (100 μM) (Bobak et al., 1990). In agreement, the concentration of GTP required for activation of the NAD:agmatine ADP-ribosyltransferase was considerably higher in SDS than in DMPC/cholate (Bobak et al., 1990). In these studies, the effect of SDS was noted at a concentration below the critical micelle concentration which is 8.6 or 11 mM at 10 or 65 °C, respectively. A stimulatory effect of SDS at submicellar concentrations has been observed in other systems. In the range of 50–100 μM, SDS activates NADPH oxidase from human macrophages (Bromberg & Pick, 1985; Pick et al., 1987) and neutrophils (Curnutte et al., 1987; Seifert & Schultz, 1987), when NADPH-dependent superoxide formation is measured, protein kinase C from rat brain (Murakami et al., 1986), and pyruvate oxidase, a flavoprotein from *Escherichia coli* (Mather & Gennis, 1985). It has been suggested that SDS at very low concentrations behaves as an ionic amphiphile (Bromberg et al., 1986). It appears that stimulation of CTA_1 auto-ADP-ribosylation by SDS results from SDS enhancement of the formation of ARF–CTA multimeric complexes that catalyze auto-ADP-ribosylation in preference to utilization of other protein substrates.

Incubation of purified ARF with GTPγS, but not GDPβS, in the presence of DMPC/cholate led to recovery of a small amount of aggregated ARF which was much more active in stimulating toxin-catalyzed ADP-ribosylation of albumin than was the monomeric ARF. When CTA was included in the incubation, the fractions containing aggregated ARF had some activity toward $G_{s\alpha}$ and albumin, indicating the presence of a trace of toxin although CTA_1 was not seen on gels. CTA_1 auto-ADP-ribosylation was not detected. Addition of ARF with or without SDS had little effect, but CTA markedly increased modification of $G_{s\alpha}$, albumin, and phosphorylase *b*, consistent with the presence of relatively more ARF than CTA in these fractions. Even when CTA (with or without SDS) was added, however, there was no auto-ADP-ribosylation with the aggregates formed in the presence of DMPC/cholate. The ARF aggregates and/or monomers are not a result of the purification procedure, since both forms, with their differences in capacity to enhance ADP-ribosylation of albumin and agmatine, were also found after incubation of crude ARF in bovine brain cytosol with DMPC/cholate and GTPγS.

Following incubation of ARF and CTA (present in molar excess of ARF) with SDS and GTPγS, only a small fraction of each protein was recovered with the aggregated complexes that preferentially catalyze auto-ADP-ribosylation of CTA_1.

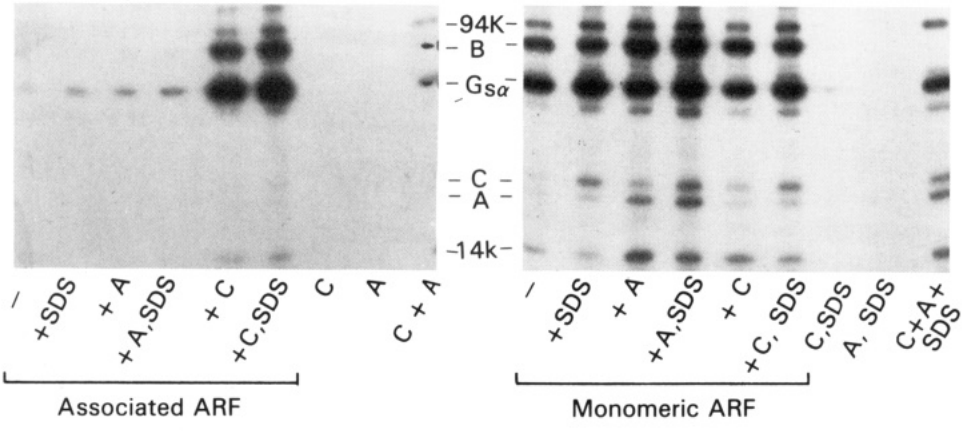

FIGURE 8: ADP-ribosylation of proteins by associated ARF formed in the presence of CTA, GTPγS, and DMPC/cholate and by monomeric proteins. From an experiment like that in Figure 7, fractions 5–8 containing ARF and a trace amount of CTA and fractions 28–32 containing monomeric ARF and CTA were separately pooled. Samples (50 μL) of each pool were incubated as described in Figure 4.

These complexes, however, contained ARF that was capable of stimulating the ADP-ribosylation of $G_{s\alpha}$ by added CTA, and toxin that preferentially catalyzed the ADP-ribosylation of $G_{s\alpha}$ in response to additional ARF. Thus, it would appear that neither the ARF nor the toxin present in the complex was saturated with the other component; both responded to addition of the other, and the response, instead of being enhanced auto-ADP-ribosylation, was expressed as increased ADP-ribosylation of $G_{s\alpha}$. Thus, such aggregates could function in the intoxication of cells by choleragen. The bulk of the ARF and CTA remained apparently monomeric and, although active, incapable of forming stable aggregated complexes on further incubation with GTPγS and SDS as if a minor component of the ARF and/or CTA with the capacity to form these complexes was used up in the initial complex formation. Auto-ADP-ribosylation by the monomeric pool was minimal compared to modification of other protein substrates. It was increased somewhat by addition of SDS and ARF or CTA but was still less than that observed with much smaller amounts of toxin and ARF in the stable aggregate formed in the presence of SDS. The aggregates, in contrast to the monomer mixture, contained ARF and toxin capable of both enhanced auto-ADP-ribosylation and increased ADP-ribosylation of $G_{s\alpha}$.

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