Activation of Immobilized, Biotinylated Choleragen A1 Protein by a 19-Kilodalton Guanine Nucleotide-Binding Protein

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ABSTRACT: Cholera toxin catalyzes the ADP-ribosylation that results in activation of the stimulatory guanine nucleotide-binding protein of the adenylyl cyclase system, known as G_s. The toxin also ADP-ribosylates other proteins and simple guanidino compounds and auto-ADP-ribosylates its A1 protein (CTA1). All of the ADP-ribosyltransferase activities of CTA1 are enhanced by 19–21-kDa guanine nucleotide-binding proteins known as ADP-ribosylation factors, or ARFs. CTA1 contains a single cysteine located near the carboxy terminus. CTA1 was immobilized through this cysteine by reaction with iodoacetyl-*N*-biotinylhexylenediamine and binding of the resulting biotinylated protein to avidin–agarose. Immobilized CTA1 catalyzed the ARF-stimulated ADP-ribosylation of agmatine. The reaction was enhanced by detergents and phospholipid, but the fold stimulation by purified sARF-II from bovine brain was considerably less than that observed with free CTA. ADP-ribosylation of $G_{s\alpha}$ by immobilized CTA1, which was somewhat enhanced by sARF-II, was much less than predicted on the basis of the NAD:agmatine ADP-ribosyltransferase activity. Immobilized CTA1 catalyzed its own auto-ADP-ribosylation as well as the ADP-ribosylation of the immobilized avidin and CTA2, with relatively little stimulation by sARF-II. ADP-ribosylation of CTA2 by free CTA1 is minimal. These observations are consistent with the conclusion that the cysteine near the carboxy terminus of the toxin is not critical for ADP-ribosyltransferase activity or for its regulation by sARF-II. Biotinylation and immobilization of the toxin through this cysteine may, however, limit accessibility to $G_{s\alpha}$ or sARF-II, or perhaps otherwise reduce interaction with these proteins whether as substrates or activator.

Cholera toxin, a secretory protein of *Vibrio cholerae* responsible for the diarrheal syndrome of cholera, causes abnormalities in intestinal fluid and electrolyte flux by activating adenylyl cyclase, thereby increasing intracellular cyclic AMP levels (Finkelstein, 1973; Kelly, 1986; Carpenter, 1980). Activation of adenylyl cyclase results from toxin-catalyzed ADP-ribosylation of G_s,[1] the stimulatory guanine nucleotide-binding (or G) protein, which is normally under the control of cell-surface receptors [for review, see Birnbaumer et al. (1987), Casey and Gilman (1988), and Moss and Vaughan (1988)]. G_s is composed of α, β and γ subunits; the α subunit, which possesses both guanine nucleotide binding and ADP-ribosylation sites, is responsible for direct activation of adenylyl cyclase when dissociated from $\beta\cdot\gamma$. The α subunit is active when GTP, but not GDP, is bound. Inactivation results from the hydrolysis of bound GTP to GDP by the intrinsic GTPase of the α subunit.

Cholera toxin catalyzed ADP-ribosylation is enhanced by a family of 19–21-kDa proteins, termed ADP-ribosylation factors or ARFs, which also bind guanine nucleotides (Kahn & Gilman, 1984, 1986; Tsai et al., 1987, 1988). These proteins, in the presence of GTP but not GDP, directly activate the A1 protein of the toxin (CTA1), enhancing its ability to catalyze the ADP-ribosylation of $G_{s\alpha}$ and other proteins unrelated to the cyclase system as well as auto-ADP-ribosylation of the A1 protein (Tsai et al., 1987, 1988).

Cholera toxin is an oligomeric protein of ~84 kDa, consisting of one A and five B subunits; the B subunits bind the toxin to the cell surface, while the A subunit is responsible for activation of adenylyl cyclase (Moss & Vaughan, 1988). The A subunit activity is latent (Mekalanos et al., 1979). Activation requires selective proteolytic cleavage to produce polypeptide protein fragments, CTA1 (23 kDa) and CTA2 (6 kDa), linked through a single disulfide bond that is located between two cysteines, one at the carboxy terminus of A1 and the other at the amino terminus of A2 and reduction of the disulfide bond (Mekalanos et al., 1979, 1983; Moss et al., 1976). Since alkylation with iodoacetamide does not block ADP-ribosyltransferase activity, a free sulfhydryl in CTA1 is not required (Moss et al., 1979).

Amino acid sequence similarities between CTA1 and pertussis toxin, another ADP-ribosyltransferase which has been studied by in vitro mutagenesis, suggest that the location of the catalytic site, at least in part, is near the amino terminus (Nicosia et al., 1986; Locht & Keith, 1986; Cieplak et al., 1988). If this were the case, it might be possible to cross-link CTA1 to a solid support through the cysteine near the carboxy terminus (which is the only cysteine in CTA1) without significantly altering catalytic activity. As reported here, CTA1 immobilized in this way is catalytically active; it differs from the soluble protein, however, in substrate specificity and responsiveness to ARF activation.

Experimental Procedures

Materials

Avidin–agarose, which bound 2.7 mg of biotin/mL of packed gel, and iodoacetyl-LC-biotin (iodoacetyl-*N*-

[1] Abbreviations: CTA, A subunit of cholera toxin; CTA1, A1 protein derived from the cholera toxin A subunit; CTA2, A2 protein derived from the toxin A subunit; immobilized CTA1 or CTA1–biotin–avidin–agarose, CTA1 protein linked covalently to biotin bound to avidin–agarose; ARF, ADP-ribosylation factor; sARF-II, one of two soluble ADP-ribosylation factors purified from bovine brain; SDS, sodium dodecyl sulfate; G_s, stimulatory guanine nucleotide binding subunit of the adenylyl cyclase system; $G_{s\alpha}$, α subunit of G_s; PAG, polyacrylamide gel; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; DMPC, dimyristoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid.

biotinylhexylenediamine) were purchased from Pierce; DMSO, DMPC, GTP, NAD, ovalbumin, and agmatine were from Sigma; SDS was from Bio-Rad; PD-10 columns were from Pharmacia; [*adenine*-U-^{14}C]NAD (285 Ci/mol) and [*carbonyl*-^{14}C]NAD (30–60 Ci/mol) were from Amersham; cholera toxin and its A subunit (CTA) were from List Biologicals.

Methods

Preparation of CTA1–Biotin–Avidin–Agarose. CTA (1 mg) suspended in 1 mL of 100 mM Tris·Cl$^-$ (pH 8)/2 mM DTT/200 mM NaCl/3 mM NaN_3/1 mM EDTA was incubated for 30 min at 30 °C. After addition of 1 mL of 10 mM iodoacetyl-LC-biotin in 20% DMSO and incubation for 2.5 h at 30 °C, the reaction was terminated with the addition of 40 μL of 1 M DTT followed by incubation for 15 min at 30 °C. Two milliliters of the mixture was transferred to a column (9 mL) of PD-10 equilibrated and eluted with 50 mM Tris·Cl$^-$, pH 8.5. After the first 2.9 mL of eluate was discarded, 2.5 mL containing CTA1–biotin and CTA2–biotin (125 μg/mL) was collected. Of this, 2.4 mL (300 μg of protein) was mixed with avidin–agarose (4 mL of packed gel) which had been equilibrated by shaking for 10 h at 4 °C with 10 volumes of 50 mM Tris·Cl$^-$ (pH 8.5)/500 mM NaCl/250 mM sucrose/1 mM NaN_3 containing ovalbumin, 0.5 mg/mL. After incubation overnight at 4 °C, the gel was washed 7 times, each with 10 mL of TENS buffer [20 mM Tris·Cl$^-$ (pH 8.0)/1 mM EDTA/1 mM NaN_3/250 mM sucrose] by shaking for 10 min at 4 °C followed by centrifugation (1000*g*, 1 min). No NAD:agmatine ADP-ribosyltransferase activity was detected in the final wash (80-μL sample). The washed gel was stored as a 50% slurry in TENS buffer.

After incubation of avidin–agarose with CTA and washing as descirbed above, essentially no NAD:agmatine ADP-ribosyltransferase activity was associated with the gel, or in the final wash (Table ID). Likewise, biotin–avidin–agarose (i.e., avidin–agarose that had been incubated with biotin before incubation with CTA1–biotin) had minimal activity in the presence of sARF-II, and there was insignificant activity in the final wash of the gel (Table IE). Thus, it appeared that ADP-ribosyltransferase activity was bound to the gel as a result of the specific interaction of CTA1–biotin with the immobilized avidin.

NAD:Agmatine ADP-Ribosyltransferase Assay. Assays performed essentially as described previously (Moss & Vaughan, 1977) contained 50 mM potassium phosphate (pH 7.5), 5 mM $MgCl_2$, 100 μM GTP, 100 μM [*adenine*-U-^{14}C]NAD (60 000 cpm), 20 mM DTT, 10 mM agmatine, and ovalbumin (0.1 mg/mL) with or without 0.003% SDS and sARF-II as indicated in a total volume of 0.3 mL. Reaction was initiated with addition of toxin or avidin–agarose preparation and incubated at 30 °C for 60 min unless otherwise indicated. When agarose was used in any of the assays, all samples were centrifuged at 4 °C (1 min, 1000*g*) before transfer of two 0.05-mL samples of supernatant to columns (0.5 × 2 cm) of Dowex AG 1-X2 (Bio-Rad) which were eluted 4 times with 1.25 mL of water. Eluates were pooled for radioassay. Data reported are means of values from duplicate assays in the same experiment. All experiments were repeated at least twice with similar results.

NAD:$G_{s\alpha}$ ADP-Ribosyltransferase Activity. Assays containing 30 mM potassium phosphate (pH 7.5), 20 mM thymidine, 250 μM GTP, 5 mM $MgCl_2$, 20 mM DTT, 50 μM [^{32}P]NAD (16 × 10^6 cpm), 1 mM DMPC, and G_s [0.2 μg in 5 μL of 200 mM potassium phosphate (pH 8.0)/0.3% sodium cholate] with or without sARF-II, 2.5 μg (final volume 100 μL), were incubated at 30 °C for the indicated times before centrifugation (1000*g*, 1 min) to sediment immobilized CTA1 before removal of samples (60 μL) for SDS–PAGE.

Protein Determination. Protein was determined by a dye-binding assay (Bio-Rad) using bovine serum albumin as a standard.

Isolation of $G_{s\alpha}$. $G_{s\alpha}$ was purified by the procedure of Sternweis et al. (1981).

SDS–PAGE. SDS–PAGE was performed as described by Laemmli (1970) with modifications. Samples (60 μL) plus 60 μL of SDS mix (203 mM Tris base/16% glycerol/1.63% SDS/16.3% mercaptoethanol/0.0025% bromophenol blue) were heated (60 °C) for 10 min and subjected to electrophoresis in 15% gels. Details are included in the figure legends. Standard proteins (Pharmacia) were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa).

Separation of Multi- and Mono-ADP-Ribosyl-CTA1 and Unmodified CTA1 by Triton X-100 Preparative Polyacrylamide Gel Electrophoresis. CTA [1.0 mg in 1.2 mL of 50 mM Tris (pH 7.5)/1 mM EDTA/3 mM NaN_3/0.2 M NaCl] was incubated overnight at 30 °C with 1 mM [^{32}P]NAD (200 μCi) in 50 mM potassium phosphate (pH 7.5)/5 mM $MgCl_2$/100 μM GTP/20 mM DTT/0.1% Triton X-100 (total volume 2 mL) followed by centrifugation (1000*g*, 5 min). The supernatant (1.95 mL) was applied to a prepacked PD-10 column (Pharmacia) equilibrated and eluted with 50 mM Tris·Cl$^-$, pH 7.5. The first 2.3 mL of eluate was discarded. The next 2.0 mL was collected; 0.85 mL was mixed with 85 μL of 1% Triton X-100, 200 μL of 50% glycerol, and 10 μL of 0.1% bromophenol blue and applied to an 8% polyacrylamide preparative gel (1.2 cm × 9 cm) (Savant Instruments) containing 0.1% Triton X-100. Electrophoresis was begun at 200 V and 36 mA; settings after elution of dye front were 200 V and 19 mA. Fractions (1 mL) were collected in 25 mM Tris·glycine, pH 8.5. To confirm the extent of ADP-ribosylation and radiolabeling, samples (80 μL) of each fraction were mixed with 8 μL of 1% Triton X-100 and 5 μL of 50% glycerol and subjected to electrophoresis in 8% polyacrylamide slab gels containing 0.1% Triton followed by autoradiography (Moss et al., 1980).

Results

In the presence of sARF-II, the NAD:agmatine ADP-ribosyltransferase activity of CTA1–biotin (Table IB) was apparently similar to that of CTA (Table IA), but activity in its absence was greater; i.e., sARF-II increased the activity of CTA 500% but that of CTA1–biotin only 100%. Similarly, the activity of CTA1–biotin–avidin–agarose was increased 150% by sARF-II (Table IC). As the amount of CTA1–biotin bound to the avidin–agarose is unknown, its activity relative to that of CTA1–biotin cannot be estimated.

CTA activity was increased slightly by DMPC/cholate, but unaffected by 0.003% SDS, whereas sARF-II activation of CTA was enhanced to a greater extent by SDS than by DMPC/cholate (Table II). Similarly, activation of immobilized CTA1 by sARF-II was enhanced more by SDS than by DMPC/cholate, neither of which had an appreciable effect in the absence of sARF-II (Table II). As shown in Table I, ARF activation of immobilized CTA1 was significantly less than was activation of free CTA (Table II).

To determine whether ADP-ribosyltransferase activity was released from immobilized CTA1 during assays, samples of medium separated from the gel after 1 h of incubation were incubated for 1 h at 30 °C while the remaining medium plus

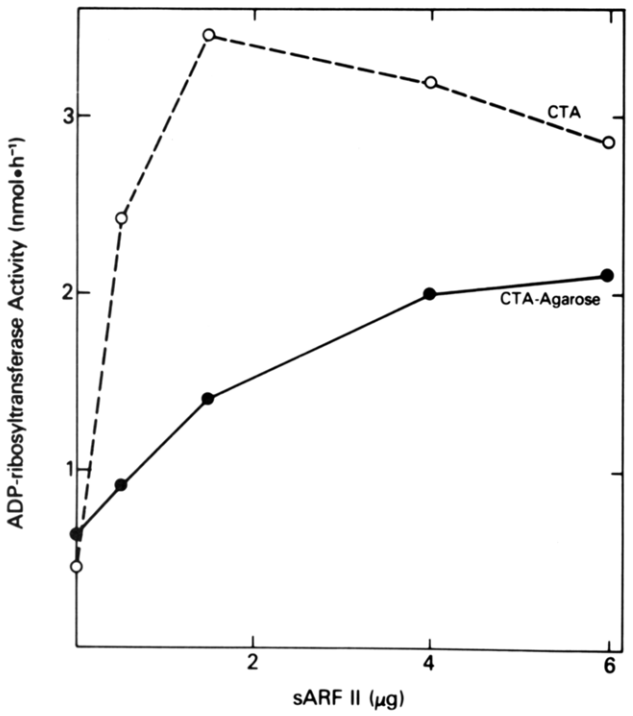

FIGURE 1: Effect of sARF-II on NAD:agmatine ADP-ribosyltransferase activities of CTA and immobilized CTA1. CTA [0.5 μg in 2.5 μL of 40 mM Tris·Cl^- (pH 7.5)/0.8 mM EDTA/2.4 mM NaN_3/160 mM NaCl] or immobilized CTA1 (CTA–agarose), 80 μL of 50% slurry in TENS buffer, was incubated in the standard NAD:agmatine ADP-ribosyltransferase assay containing 50 mM potassium phosphate, pH 7.5, 5 mM $MgCl_2$, 100 μM GTP, 100 μM [*adenine*-U-^{14}C]NAD (60000 cpm), 20 mM DTT, 10 mM agmatine, 0.003% SDS, and 0.1 mg/mL ovalbumin (total volume 0.3 mL). Data are means of values from duplicate assays. Activity (nmol·h^{-1}) of the CTA1–biotin (2.1 μg) in the absence of sARF-II was 2.23, and in the presence of 4 and 6 μg of sARF-II was 10.1 and 10.8, respectively.

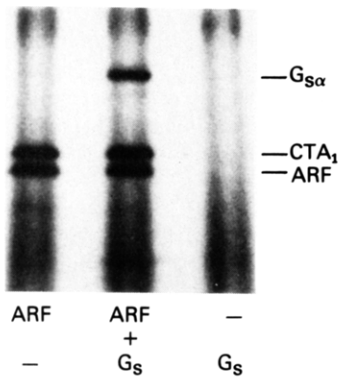

FIGURE 2: Effect of sARF-II on [^{32}P]ADP-ribosylation of $G_{s\alpha}$ by CTA. Assays containing CTA (0.5 μg in 2.5 μL of 40 mM Tris·Cl^-, pH 7.5, 0.8 mM EDTA, 2.4 mM NaN_3, and 160 mM NaCl) and other components as described under Experimental Procedures were incubated for 1 h at 30 °C.

gel was further incubated for 1 h. In the latter samples, ADP-ribosylagmatine formation continued during the second hour while there was no further production in medium separated from the gel (Table II). The data demonstrate the stability of toxin binding to the gel under assay conditions.

ARF activation of immobilized CTA1 (like that of CTA) was GTP-dependent and enhanced by SDS (Table III). Comparing CTA and immobilized CTA1 at similar levels of basal activity, less sARF-II was required to achieve maximal activity of CTA, and maximal activation of CTA was considerably greater (Figure 1).

Table I: Effect of sARF-II on ADP-Ribosyltransferase Activity of CTA, CTA1–Biotin, and CTA1–Biotin–Avidin–Agarose[a]

toxin or agarose preparation	ADP-ribosyltransferase act. (nmol) no sARF-II	0.75 μg of sARF-II
(A) CTA, 0.2 μg	0.15	0.94
(B) CTA1–biotin, 2.5 μg	4.9	10.5
(C)[b] CTA1–biotin–avidin–agarose, 40 μL	0.6	1.61
(D)[b] avidin–agarose, 40 μL, previously incubated with CTA	0.03	0.09
(E)[b] biotin–avidin–agarose, 40 μL, previously incubated with CTA1–biotin	0.02	0.12

[a] NAD:agmatine ADP-ribosyltransferase activity was assayed as described under Methods with and without sARF-II, 0.75 μg. Reactions were initiated with (A) CTA [in 2.5 μL of 16 mM Tris·Cl^- (pH 7.5)/0.2 mM EDTA/0.6 mM NaN_3/40 mM NaCl], (B) CTA1–biotin (20 μL of solution prepared as described under Methods), and (C–E) the indicated avidin–agarose preparation (added as 80 μL of a 50% slurry in TENS buffer). (D) For preparation of avidin–agarose incubated with CTA, 1 mL of avidin–agarose (packed gel) was shaken overnight at 4 °C with 0.25 mg of CTA in 1 mL of 50 mM Tris (pH 7.4)/1 mM EDTA/3 mM NaN_3/0.2 M NaCl/2 mM DTT incubated for 15 min at 30 °C before addition. The gel was washed 7 times with 14 mL each of TENS buffer by shaking 10 min at 4 °C followed by centrifugation (1000*g*, 1 min) and stored as a 50% slurry in TENS buffer. (E) For preparation of biotin–avidin–agarose incubated with CTA1–biotin, 1 mL of avidin–agarose (packed gel) was shaken with 100 mg of biotin in 4 mL of TENS buffer for 4 h at 4 °C (final volume 5 mL) with shaking and centrifuged (1000*g*, 1 min) before removal of supernatant and addition of 0.6 mL of CTA–biotin (125 μg/mL) in 50 mM Tris·Cl^-, pH 8.5. After incubation overnight at 4 °C, the gel was washed 7 times, each with 14 mL of TENS buffer by shaking 10 min at 4 °C followed by centrifugation (1000*g*, 1 min). The washed gel was stored as a 50% slurry in TENS buffer. [b] Samples (80 μL) of final washes of each gel were assayed. Activities without and with ARF were, respectively, for (C) 0 and 0, for (D) 0 and 0.04, and for (E) 0.02 and 0.02.

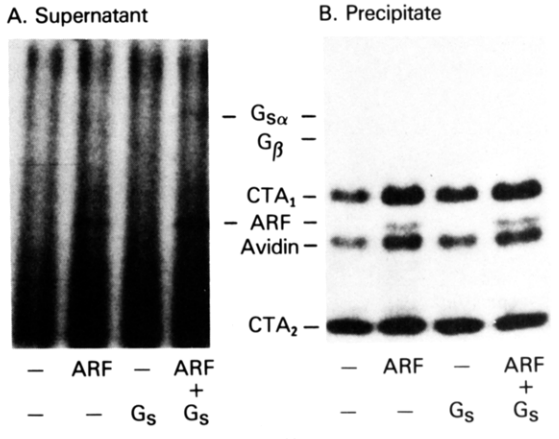

FIGURE 3: Effect of sARF-II on [^{32}P]ADP-ribosylation of $G_{s\alpha}$ and other proteins by immobilized CTA1. Assays containing immobilized CTA1 (80 μL of 50% slurry in TENS buffer) and other components as described under Experimental Procedures were incubated for 12 h at 30 °C and centrifuged to sediment the gel. (A) After centrifugation, 50 μL of supernatant was mixed with 50 μL of "SDS mix" (see Experimental Procedures), and 90 μL was subjected to electrophoresis in a 15% gel. (B) The gel was washed 3 times each with 10 mL of 50 mM Tris·Cl^- (pH 8.5)/0.2 M NaCl followed by centrifugation and boiled for 5 min with 90 μL of SDS mix. Samples (60 μL) were subjected to electrophoresis.

ARF stimulation of toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$ is enhanced by DMPC/cholate (Schleifer et al., 1982; Tsai et al., 1988), and under these conditions, there was significant auto-ADP-ribosylation of CTA1 and ADP-ribosylation of sARF-II as well as $G_{s\alpha}$ (Figure 2). Incubation of immobilized CTA1 under the same conditions, however, resulted in recovery

Table II: Effects of sARF-II, SDS, and DMPC/Cholate on the Release of NAD:Agmatine ADP-Ribosyltransferase Activity from Immobilized CTA1[a]

additions	[*adenine*-U-^{14}C]-ADP-ribosylagmatine formation (nmol/assay) after 1 h	2 h
(A) CTA (0.5 μg)	0.98	2.2
plus SDS	1.04	2.3
plus DMPC/cholate	1.24	3.1
(B) CTA plus sARF-II	2.8	9.1
plus SDS	8.0	20.3
plus DMPC/cholate	4.5	12.4
(C) immobilized CTA1, 40 μL	1.1 (1.3)[b]	3.1
plus SDS	1.3 (1.2)	3.0
plus DMPC/cholate	1.4 (1.3)	3.4
(D) immobilized CTA1, plus sARF-II	2.0 (2.1)	5.1
plus SDS	2.8 (2.7)	7.2
plus DMPC/cholate	2.4 (2.6)	6.6

[a] NAD:agmatine ADP-ribosyltransferase activity was assayed in a total volume of 0.6 mL with concentrations of all components as described under Methods and 2.5 μg of sARF-II, 0.003% SDS, or 1 mM DMPC/0.015% cholate present as indicated. Reactions were initiated with CTA [0.5 μg in 2.5 μL of 40 mM Tris·Cl$^-$ (pH 7.5)/0.8 mM EDTA/2.4 mM NaN_3/160 mM NaCl] or immobilized CTA1 (80 μL of a 50% slurry in TENS buffer). After incubation for 1 h at 30 °C and centrifugation (1 min, 1000*g*), two 0.05-mL samples of supernatant were removed for determination of [*adenine*-U-^{14}C]ADP-ribosylagmatine formed, and 0.14 mL of supernatant was incubated for 1 h at 30 °C, followed by removal of two 0.05-mL samples for determination of [*adenine*-U-^{14}C]ADP-ribosylagmatine. The gel and remaining supernatant were mixed and incubated at 30 °C for 1 h before centrifugation and removal of two 0.05-mL samples for determination of [*adenine*-U-^{14}C]ADP-ribosylagmatine. Data are means of values from duplicate assays normalized to 0.6-mL initial assay volume. [b] In parentheses, [*adenine*-U-^{14}C]ADP-ribosyltransferase in supernatant incubated for 1 h after removal of gel following incubation for 1 h.

Table III: Effects of sARF-II, GTP, and SDS on NAD:Agmatine ADP-Ribosyltransferase Activity of Immobilized CTA1[a]

additions	[*carbonyl*-^{14}C]nicotinamide released (nmol) no SDS	0.003% SDS
none	0.70	0.64
sARF-II, 2.5 μg	0.75	0.70
GTP, 100 μM	0.64	0.62
GTP plus sARF-II	0.90	1.15

[a] NAD:agmatine ADP-ribosyltransferase activity was assayed as described under Methods except that 100 μM [*carbonyl*-^{14}C]NAD (60 000 cpm) replaced the [*adenine*-U-^{14}C]NAD and the final concentrations of additions were 100 mM agmatine, 2.5 μg of sARF-II, 100 μM GTP, 0.003% SDS, and 20 mM DTT. Assays in duplicate were initiated with 60 μL of a 50% slurry of immobilized CTA1. Two 0.05-mL samples from each assay were used for determination of [*carbonyl*-^{14}C]nicotinamide released.

of relatively little ADP-ribosylated sARF-II or $G_{s\alpha}$ from the medium (Figure 3A). The gel was separated from medium at the end of the assay and boiled to release proteins that were then analyzed by SDS–PAGE and autoradiography (Figure 3B). After incubation in the absence of sARF-II or $G_{s\alpha}$, there was considerable ADP-ribosylation of a protein with mobility slightly less than that of CTA1, presumably auto-ADP-ribosylated CTA1 as well as labeled bands with mobilities expected of avidin and CTA2 (Figure 3B, first lane). sARF-II increased ADP-ribosylation of avidin and auto-ADP-ribosylation of CTA1. There were also small amounts of apparently ADP-ribosylated sARF-II (Figure 3B, second and fourth lanes). Traces of labeled $G_{s\alpha}$ were found after incubation in the presence of sARF-II (Figure 3B, fourth lane) but not in its absence (Figure 3B, third lane). Immobilization

Table IV: Effect of sARF-II on the Activity of ADP-Ribosylated CTA1[a]

toxin preparation (1 μg)	ADP-ribosyltransferase act. (nmol·μg^{-1}·h^{-1}) basal	sARF-II	sARF-II + SDS
CTA	0.95	2.4	8.0
mono-ADP-ribosylated CTA1	1.30	2.7	7.8
di-ADP-ribosylated CTA1	1.26	2.8	6.3
tri-ADP-ribosylated CTA1	0.71	1.3	5.1

[a] NAD:agmatine ADP-ribosyltransferase activity was assayed as described under Methods without (basal) or with (1 μg) sARF-II and 0.003% SDS as indicated. Reactions were initiated with CTA or mono-, di-, or tri-ADP-ribosylated CTA1 (1 μg) and incubated for 60 min at 30 °C. Data are means of values from duplicate assays.

of CTA1 apparently favored auto-ADP-ribosylation and ADP-ribosylation of the two other immobilized proteins, biotin–CTA2 and avidin, which were only slightly enhanced by sARF-II, but interfered with ADP-ribosylation of $G_{s\alpha}$ and sARF-II.

The ADP-ribosylation of biotin–CTA2 by immobilized CTA1 is striking, since, when the toxin is in solution, labeling of CTA2 is minimal. It is not known how much biotin–CTA2 is immobilized through a biotin–avidin interaction and how much through biotin–CTA2 association with biotin–CTA1. With soluble toxin, denaturation is required for separation of reduced and alkylated CTA1 and CTA2.

To determine whether auto-ADP-ribosylation of CTA1 modified its responsiveness to sARF-II, mono-, di-, and tri-ADP-ribosylated species of CTA1 were prepared. All of these were active, and activity was increased by sARF-II or sARF-II plus SDS, although the tri-ADP-ribosylated CTA1 was less active than CTA1 or the other modified forms (Table IV). Prolonged incubation of toxin with sARF-II plus SDS resulted in a species that did not readily enter the Triton X-100–polyacrylamide gels and appears to have been ADP-ribosylated to a much greater extent than those in Table IV (data not shown).

Discussion

The studies reported here support the conclusion that the cysteine and, presumably, adjacent portions of the carboxy-terminal region of CTA1 are not critical for ADP-ribosyltransferase activity or for ARF stimulation of that activity. CTA1 linked to biotin through this cysteine and immobilized via biotin binding to avidin–agarose catalyzed the ADP-ribosylation of agmatine, indicating that both the NAD and ADP-ribose acceptor sites were intact. The activity was enhanced by ARF, albeit to a lesser degree than was that of unmodified CTA1. Immobilization likewise reduced the stimulation of ADP-ribosylation of $G_{s\alpha}$ by ARF in the presence of DMPC/cholate. It appears, however, that DMPC/cholate does not uniformly inhibit association of substrate with immobilized CTA1 since DMPC/cholate did not decrease ARF stimulation of agmatine ADP-ribosylation by immobilized CTA1. In the presence of DMPC/cholate, auto-ADP-ribosylation of immobilized CTA1 was significant. It was not, however, enhanced by sARF-II to as great an extent as was auto-ADP-ribosylation of free CTA1 in DMPC/cholate. These data are consistent with the possibility that auto-ADP-ribosylation of immobilized CTA1 is an intramolecular reaction, whereas auto-ADP-ribosylation of free CTA1 may at least to some extent be intermolecular.

Mono-, di-, or tri-auto-ADP-ribosylation of free CTA1 did not appear to interfere with its responsiveness to sARF-II plus SDS. It is possible that the extent of ADP-ribosylation of

immobilized CTA1 was significantly greater and that this species was unresponsive to sARF-II. The immobilized toxin apparently catalyzed the ADP-ribosylation of both avidin and CTA2 in a reaction that was essentially sARF-II independent. In assays with soluble components, the effect of sARF-II plus SDS is to enhance both affinity and maximal velocity. It may not be surprising that its apparent stimulatory effects with immobilized enzyme (CTA1) and substrates (CTA2 and avidin) are minimal.

The observations on immobilized CTA1 may be compared with results of studies on a related ADP-ribosyltransferase, pertussis toxin, which catalyzes the ADP-ribosylation of guanine nucleotide-binding proteins involved in adenylyl cyclase inhibition, phospholipid turnover, and ion flux (Birnbaumer et al., 1987; Casey & Gilman, 1988; Moss & Vaughan, 1988). Cross-linking the enzymatically active S1 protein of pertussis toxin through a cysteine located near the carboxy terminus inhibited ADP-ribosylation of the guanine nucleotide binding proteins, but did not alter NAD glycohydrolase activity (Kaslow et al., 1989). Several sites involved in the NAD glycohydrolase and ADP-ribosyltransferase activities have been identified by immunological analysis and by in vitro mutagenesis of S1 (Nicosia et al., 1986; Locht & Keith, 1986; Cieplak et al., 1988). The amino terminus of S1 appears to be crucial for activity (Cieplak et al., 1988) and exhibits considerable homology with CTA1 (Nicosia et al., 1986; Locht & Keith, 1986; Cieplak et al., 1988). Thus, the amino terminus may be more critical to association with NAD while the carboxy end of S1 and CTA1 may participate more in the interaction with the respective G protein substrates. The ability of CTA1 when cross-linked to utilize simple guanidino compounds demonstrates that the ADP-ribose acceptor site is still intact. Since the cross-linked CTA1 is still responsive to sARF-II, it would appear that the activation site is not located in the carboxy terminus of CTA1.

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