

Mechanism of cholera toxin action: Covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system

(pigeon erythrocyte membranes/ADP-ribosylation/fluoride activation)

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ABSTRACT Treatment of pigeon erythrocyte membranes with cholera toxin and NAD^+ enhanced the GTP stimulation and suppressed the F^- activation of the adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. In the presence of NAD^+ labeled with ^{32}P in the AMP moiety the toxin catalyzed the covalent incorporation of radioactivity into membrane proteins with molecular weights (M_r s) of 200,000, 86,000, and 42,000. Extraction of toxin-treated membranes with Lubrol PX followed by affinity chromatography on a GTP-Sepharose column resulted in a 200-fold purification of the 42,000- M_r labeled protein and in its complete separation from the other labeled proteins. The fraction containing the purified GTP-binding component from toxin-treated membranes conferred an enhanced GTP-stimulated activity on adenylate cyclase solubilized from nontreated membranes. Likewise, the addition of GTP-binding fraction from nontreated membranes to an enzyme solubilized from toxin-treated membranes restored F^- stimulation of the adenylate cyclase. The toxin-induced modification of adenylate cyclase and the incorporation of radioactivity into the 42,000- M_r protein were partially reversed upon incubation with toxin and nicotinamide at pH 6.1. The results indicate that cholera toxin affects the adenylate cyclase system by catalyzing an ADP-ribosylation of the 42,000- M_r component bearing the guanyl nucleotide regulatory site.

Cholera toxin exerts its effects on the small intestine *in vivo* and on a variety of cells from other organs *in vitro* through activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] (1-4). Activation in broken cell preparations, in membranes (5-6), or in detergent-solubilized adenylate cyclase preparations (7) is dependent on the presence of the A subunit of the toxin, NAD^+ , cellular cytosolic factors, and ATP. By analogy to diphtheria toxin (8), the action of cholera toxin was assumed to involve ADP-ribosylation. However, the target for cholera toxin action in the plasma membrane has not as yet been identified. Cassel and Selinger have recently demonstrated that cholera toxin inhibits the catecholamine-stimulated GTPase activity in turkey erythrocyte membranes (9, 10). This finding was consistent with the observation that in toxin-treated membranes GTP became as effective as the nonhydrolyzable GTP analog guanosine 5'-(β,γ -imino)triphosphate [Gpp(NH)p] in stimulating the adenylate cyclase. Recent studies indicate that the hormone-stimulated adenylate cyclase consists of at least three separable components: the hormone receptor, a catalytic moiety, and a guanyl nucleotide-binding protein (11-15). Using affinity chromatography, Pfeuffer has been able to dissociate a guanyl nucleotide-binding protein from a solubilized adenylate cyclase preparation of pigeon erythrocytes. This resulted in a loss of guanyl nucleotide and F^- activation, both of which could be restored upon reconstitution (14).

We now show that a GTP-binding protein with an M_r of 42,000 is covalently modified in the presence of ^{32}P - NAD^+ and

cholera toxin and that the modification is probably an ADP-ribosylation. Cross reconstitution of the adenylate cyclase from separated regulatory and catalytic components demonstrates that the modification of the 42,000- M_r GTP binding protein is responsible for the toxin-induced changes in adenylate cyclase activity.

MATERIALS AND METHODS

[α - ^{32}P]ATP was from the Radiochemical Centre, Amersham, England. Cholera toxin was obtained from Schwarz/Mann; β -NAD, guanosine 5'-(3-thio)triphosphate (GTP γ S), and NAD pyrophosphorylase (EC 2.7.7.1) were from Boehringer. ADP-ribose was prepared from NAD by the action of *Neurospora crassa* NADase (EC 3.2.2.5, Sigma). Pigeon erythrocyte membranes were prepared and adenylate cyclase solubilized with Lubrol PX as described previously (13). Adenylate cyclase activity was assayed for 20 min at 37° according to Salomon *et al.* (17). The reaction mixture in a final volume of 0.1 ml contained [α - ^{32}P]ATP (0.5 - 2×10^6 cpm), 0.3 mM unlabeled ATP (Sigma A 2383), 6 mM MgCl_2 , 1 mM 3',5'-cyclic AMP (cAMP), 12 mM creatine phosphate, 5 units of creatine kinase, 2 mM 2-mercaptoethanol, and 25 mM 3-(*N*-morpholino)propane-sulfonic acid (Mops) buffer, pH 7.4. Protein was determined by the method of Lowry *et al.* (18) with bovine serum albumin as standard.

Toxin Treatment. Pigeon erythrocyte membranes were treated with cholera toxin essentially as described previously for turkey erythrocyte membranes (9). Cytosol was obtained by lysis of packed pigeon erythrocytes by freezing followed by thawing and resuspension in an equal volume of Mops/saline buffer (20 mM Mops, pH 7.4/130 mM NaCl/10 mM KCl/2 mM MgCl_2 /1 mM dithiothreitol). Membranes and nuclei were removed by centrifugation at $20,000 \times g$ for 10 min. The supernatant was depleted of endogenous NAD^+ by gel filtration on Sephadex G-25, using the centrifuge column procedure (19). The cytosol was preincubated with cholera toxin for 15 min at 37° and then mixed with an equal volume of pigeon erythrocyte membranes (20 mg/ml) in Mops/saline. The suspension was incubated for 30 min at 30° in the presence of 1 mM ATP/10 mM phosphoenolpyruvate/50 μg of pyruvate kinase per ml/either 1 mM unlabeled NAD^+ or 20 μM [^{32}P]NAD $^+$ (2000-6000 cpm/pmol). Membranes were separated from cytosol by centrifugation ($15,000 \times g$ for 30 min) through a sucrose cushion (25% wt/wt sucrose in Mops/saline containing 0.2% bovine serum albumin). Finally, the membrane pellet was washed twice with Mops/saline.

Abbreviations: M_r , molecular weight; Gpp(NH)p, guanosine 5'-(β,γ -imino)triphosphate; GTP γ S, guanosine 5'-(3-thio)triphosphate; cAMP, adenosine 3',5'-cyclic monophosphate; Mops, 3-(*N*-morpholino)propane-sulfonic acid; NaDodSO $_4$, sodium dodecyl sulfate.

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Preparation of [^{32}P]NAD $^{+}$. NAD $^{+}$ with the AMP moiety labeled with ^{32}P was prepared from [α - ^{32}P]ATP and nicotinamide mononucleotide (NMN) by using NAD $^{+}$ pyrophosphorylase. The reaction mixture contained 0.1–0.2 mM [α - ^{32}P]ATP (10–20 Ci/mmol), 10 mM NMN, 0.1 unit of NAD $^{+}$ -pyrophosphorylase per ml, 10 mM MgCl $_2$, and 25 mM Mops buffer, pH 7.4. After 30-min incubation at 37° the reaction was stopped by immersion of the mixture in a boiling water bath for 1 min. The denatured enzyme was removed by centrifugation and the supernatant was loaded on a column (0.8 × 4 cm) filled with Dowex 1-X8 (200–400 mesh) in the formate form. Elution was with 0.1 M formic acid. The first 4.5 ml of the eluate (containing the NMN) was discarded and [^{32}P]NAD $^{+}$ was eluted in the following 6 ml. This fraction was evaporated to dryness under reduced pressure and the [^{32}P]NAD was resuspended in 50–100 μl of water. The yield of [^{32}P]NAD was 60–70%, based on [α - ^{32}P]ATP. The preparation was 95–98% radiochemically pure as determined by chromatography on polyethyleneimine-cellulose.

Nucleotides were chromatographed on polyethyleneimine-cellulose sheets developed in 0.15 M NH $_4$ HCO $_3$ (R_F values: NAD $^{+}$, 0.54; AMP, 0.30; ADP, 0.10; ADP-ribose, 0.34) or in 0.1 M LiCl/0.5 M formic acid (R_F values: NAD, 0.80; AMP, 0.77; ADP, 0.16; ADP-ribose, 0.47). The preparation of the GTP-Sepharose derivative is described in ref. 14.

RESULTS

Effects of Cholera Toxin on Adenylate Cyclase Activity and Their Relation to the Covalent Modification of Membrane Proteins. Pigeon erythrocyte membranes, treated with cholera toxin in the presence of [^{32}P]NAD $^{+}$, were analyzed by sodium dodecyl sulfate (NaDodSO $_4$)/polyacrylamide gel electrophoresis. As shown in Fig. 1, essentially three proteins were labeled with M_r values of \sim 200,000, 86,000, and 42,000.

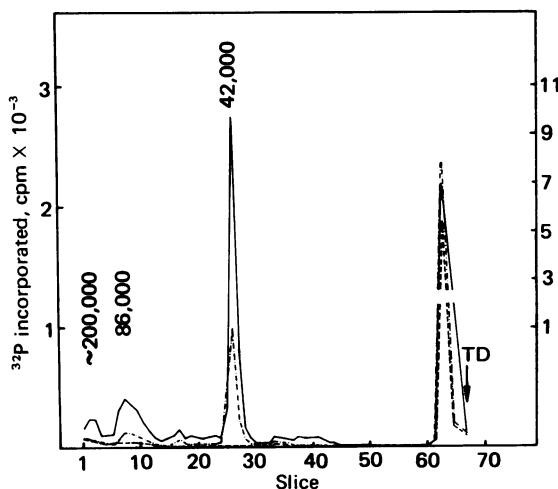


FIG. 1. NaDodSO $_4$ /polyacrylamide gel electrophoresis of pigeon erythrocyte membranes treated with cholera toxin and [^{32}P]NAD $^{+}$. Membranes were incubated with 20 μM [^{32}P]NAD $^{+}$ (5000 cpm/pmol) and the additions stated in *Materials and Methods* for 30 min at 30° with cholera toxin at 0 (---), 8 (---), or 50 (—) $\mu\text{g}/\text{ml}$. After washing, the membranes (0.2 mg protein) were dissociated with 1% NaDodSO $_4$ and 1% (vol/vol) 2-mercaptoethanol for 30 min at 37°. Polyacrylamide gel electrophoresis (13% gels) in the presence of 0.1% NaDodSO $_4$ was carried out according to Neville and Glossmann (20). The gels were cut into 1-mm discs and assayed for radioactivity in 3 ml of Bray's solution (21). Molecular weights were estimated using the following proteins for comparison: soybean trypsin inhibitor, M_r 21,000; ovalbumin, M_r 45,000; bovine serum albumin, M_r 68,000; and rabbit skeletal muscle phosphorylase b, M_r 100,000. TD, tracking dye.

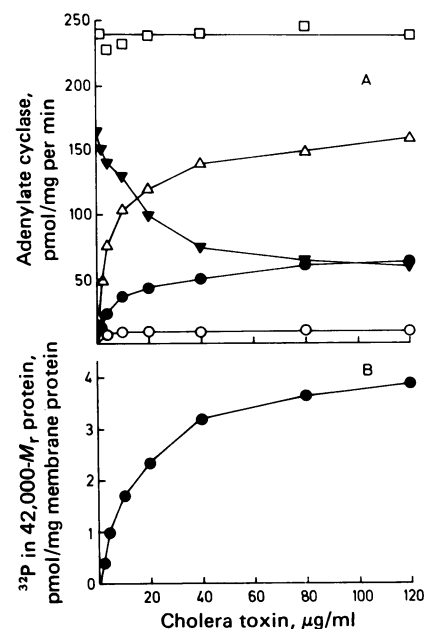


FIG. 2. Correlation of changes of adenylate cyclase activity with the covalent labeling of the 42,000- M_r protein. Membranes were treated with various toxin concentrations in the presence of 20 μM [^{32}P]NAD $^{+}$. (A) Adenylate cyclase activity: O, no additions; ●, with 0.1 mM GTP; Δ, with 0.1 mM GTP and 50 μM isoproterenol; ▼, with 10 mM NaF; □, with 10 μM GTP γ S plus 50 μM isoproterenol. (B) Radioactivity in the 42,000- M_r protein determined by NaDodSO $_4$ /polyacrylamide gel electrophoresis (see Fig. 1).

The radioactive peak following the tracking dye represents free [^{32}P]NAD $^{+}$. No radioactivity was incorporated into membranes in the absence of cholera toxin. Recent studies by Pfeuffer (14) have indicated that a protein with M_r 42,000 is the GTP-binding component associated with adenylate cyclase. A protein with the same M_r became labeled by [^{32}P]NAD $^{+}$ upon exposure of membranes to cholera toxin. It was therefore tested whether the covalent modification of this protein correlates with the toxin-induced changes in adenylate cyclase activity. The response of adenylate cyclase from pigeon erythrocyte membranes to cholera toxin turned out to be similar to that previously described for the turkey erythrocyte adenylate cyclase (9): at relatively low toxin concentrations, the most significant change was the increase in the activation by GTP with or without isoproterenol. On the other hand, the toxin had no effect on the activation by GTP γ S in the presence of isoproterenol. GTP γ S, in contrast to GTP, is not hydrolyzed at the regulatory site (14, 22).[‡] At somewhat higher toxin concentrations F $^{-}$ -stimulated activity was suppressed (Fig. 2A). Like the changes in adenylate cyclase activity, the incorporation of ^{32}P into the 42,000- M_r protein approached saturation at a toxin concentration of about 40 $\mu\text{g}/\text{ml}$. With 20 μM NAD $^{+}$ [§] the incorporation leveled off at about 4 pmol of ^{32}P per mg of

[‡] GTP γ S rather than Gpp(NH)p was used as a hydrolysis-resistant GTP analog because GTP γ S gave maximal activation of the adenylate cyclase of both the toxin-treated and nontreated preparations, whereas the activation by Gpp(NH)p was significantly reduced after toxin treatment (see also ref. 9).

[§] In order to achieve high enough specific radioactivity, low [^{32}P]NAD $^{+}$ concentration (20 μM) was used for the comparative studies in Fig. 2. At this low NAD $^{+}$ concentration, the modification of adenylate cyclase activity by the toxin was incomplete. At saturating NAD $^{+}$ concentration (1 mM), F $^{-}$ stimulation was completely abolished and the activation by GTP and isoproterenol reached a maximum of 175 pmol/mg per min.

membrane protein (see Fig. 2B), whereas with 0.1 mM NAD⁺, 8 pmol of ³²P per mg was incorporated (not shown). At toxin concentration <40 μg/ml, the extent of covalent modification was better correlated with the sum of the changes in GTP and F⁻ stimulation, rather than with either of the effects alone. This might mean that cholera toxin catalyzes the covalent modification of two sites each on a protein with 42,000 M_r, resulting in the modification of the two different functions of the adenylate cyclase.

We have attempted to identify the proteins labeled with [³²P]NAD⁺ and cholera toxin in turkey erythrocyte membranes, but have failed. Incubation of purified turkey erythrocyte membranes with [³²P]NAD⁺ in the absence of cholera toxin resulted in the covalent incorporation of about 2 nmol of ³²P per mg of membrane protein, and this high level of incorporation precluded the detection of covalently modified proteins in these membranes on cholera toxin treatment. A possible explanation for the large toxin-independent incorporation could be an ADP-ribosylation of membrane proteins by an NADase, because this enzyme can transfer ADP-ribose to acceptors besides H₂O (23). Turkey erythrocytes, in contrast to pigeon erythrocytes, are known to contain NADase activity (5).

Purification of the Covalently Labeled 42,000-M_r Protein by Affinity Chromatography on GTP-Sepharose. The enhancement of GTP stimulation and the decrease of fluoride-stimulated adenylate cyclase activity in toxin-treated membranes were also observed with an adenylate cyclase preparation solubilized with Lubrol PX from toxin-treated membranes (see Table 1). Noteworthy is the activation of the soluble preparation from toxin-treated membranes by GTPγS. Previous work (13, 14) has shown that adenylate cyclase solubilized from pigeon erythrocyte membranes is only marginally activated by nonhydrolyzable GTP analogs such as Gpp(NH)p and GTPγS unless the membranes have been incubated with isoproterenol and GMP before solubilization. This contrasts strikingly with the response of adenylate cyclase solubilized from cholera toxin-treated membranes, which was markedly stimulated by GTPγS even without prior treatment of the membranes with GMP and isoproterenol (Table 1). The GTP stimulation of the toxin-treated preparation was 1/5th that of the GTPγS stimulation of the same preparation. This is probably due to the fact that the toxin-induced inhibition of GTP hydrolysis at the regulatory site is incomplete (9). Solubilization of membranes pretreated with [³²P]NAD⁺ (20 μM) in the presence of cholera toxin, with Lubrol PX, resulted in the extraction of 50% of the 86,000-M_r labeled protein and 70% of

Table 1. Comparison of soluble adenylate cyclase activity from control and toxin-treated membranes

Additions to the assay	Adenylate cyclase solubilized, pmol cAMP/mg per min	
	Nontreated membranes	Toxin-treated membranes
None	11	40
GTP	10	127
GTPγS	54*	605*
F ⁻	529	42

Membranes were treated with cholera toxin (5 μg/ml) and NAD⁺ (1 mM) and then solubilized with Lubrol PX. Control membranes were processed identically but in the absence of toxin. The concentrations of GTP, GTPγS, and F⁻ in the adenylate cyclase assay were 0.1 mM, 10 μM, and 8 mM, respectively.

* Adenylate cyclase solubilized from membranes preincubated with isoproterenol and GMP and assayed with GTPγS had an activity of about 1000 pmol of cAMP formed per mg per min in both toxin-treated and nontreated membranes.

Table 2. Separation of ³²P-labeled membrane proteins on GTP-Sepharose

Fractions	³² P incorporation (cpm) into proteins with M _r		Protein, μg
	86,000	42,000	
I. Unfractionated system	644	2,040	120
II. Fraction not retained by GTP-Sepharose	600	920	115
III. Wash fluid	20	30	—
IV. Fraction released with GTPγS from the GTP-Sepharose	9	1,030	0.9

Membranes were treated with cholera toxin (40 μg/ml) and [³²P]NAD⁺ (20 μM). Washed membranes (5 mg/ml) were then treated with GMP and isoproterenol and solubilized with 20 mM Lubrol PX (13). A sample (250 μl) of the soluble preparation (1.2 mg/ml) was chromatographed at 22° on a GTP-Sepharose 4B column (120-μl bed volume, equilibrated with 1 mM MgEDTA/1 mM 2-mercaptoethanol/30 mM NaCl/10 mM Mops buffer, pH 7.4). The column was rinsed with an additional 250 μl of the same buffer containing 90 mM NaCl. This eluent (fraction II) contains the material not adsorbed to the column. The column was then washed with an additional 500 μl of the same buffer (fraction III). The GTP-binding proteins were released by the slow passage (45 min) of 500 μl of buffer containing 0.2 mM GTPγS (fraction IV). One hundred microliters of the material applied to the column (fraction I) and 200 μl of the effluents (fractions II-IV) were treated with NaDodSO₄ and 2-mercaptoethanol and subjected to polyacrylamide gel electrophoresis in the presence of NaDodSO₄ as described in Fig. 1.

the 42,000 M_r labeled protein. The protein with M_r ~ 200,000 was not extracted by the detergent (not shown).

The radioactively labeled detergent-solubilized preparation was chromatographed on a GTP-Sepharose affinity column (Table 2). Preparation and application of this affinity matrix were described in detail recently (14). For affinity chromatography, cholera toxin-treated membranes were incubated with isoproterenol and GMP prior to solubilization, because this treatment was previously found necessary for binding of the solubilized GTP-binding component of the adenylate cyclase to the GTP-Sepharose derivative (14). Table 2 and Fig. 3 show that about 50% of the covalently labeled 42,000-M_r protein was selectively retained by the GTP-Sepharose. This protein was not eluted with buffer, but was quantitatively released by a

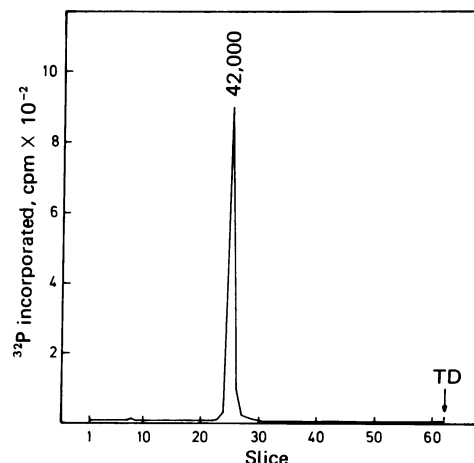


FIG. 3. NaDodSO₄/polyacrylamide gel electrophoresis of ³²P-labeled protein purified by affinity chromatography (fraction IV in Table 2). TD, tracking dye.

Table 3. Cross-reconstitution of the adenylate cyclase from separated catalytic and regulatory components of nontreated and cholera toxin-treated membranes

Acceptor preparations depleted of GTP-binding proteins	Effectors in assay	Activity (pmol cAMP per assay) with donor preparations used for reconstitution		
		No addition	GTP-binding components	
			Nontreated membranes	Toxin-treated membranes
I. From nontreated membranes	GTP	13*	16	122
	GTP + F ⁻	330*†	630	369
II. From toxin-treated membranes	GTP	99*	103	217
	GTP + F ⁻	132*	552	161
III. Buffer	GTP	—	3	1
	GTP + F ⁻	—	8	2

Membranes treated with or without cholera toxin (5 µg/ml) plus NAD⁺ (1 mM) were incubated with GMP and isoproterenol and solubilized as described in the legend to Table 2. Affinity chromatography on GTP-Sepharose 4B was performed as described in the legend to Table 2 and in ref. 14 with the exception that the GTP-binding protein was eluted with 0.2 mM GTP instead of GTPγS. "Depleted" adenylate cyclase refers to the material not retained by GTP-Sepharose. For reconstitution, depleted adenylate cyclase (50 µg of protein) was combined with GTP-binding fractions (either 2 µg of protein for GTP-stimulated activity or 1 µg of protein for stimulation with GTP + F⁻) and immediately assayed for adenylate cyclase activity. Unfractionated adenylate cyclase refers to a soluble adenylate cyclase preparation that was passed over carboxypropylamino-Sepharose 4B [intermediate of GTP-Sepharose (14)] under identical conditions. In the control samples, i.e., the unfractionated adenylate cyclase and the depleted preparations, corrections were made for changes in volume and detergent to protein ratios (14). Final concentrations of effectors in the assay were: 0.1 mM GTP or 0.1 mM GTP plus 8 mM NaF.

* The activities (pmol/assay) of unfractionated adenylate cyclase preparations were: nontreated enzyme: 10 with GTP, 715 with GTP + F⁻; toxin-treated enzyme: 215 with GTP, 270 with GTP + F⁻.

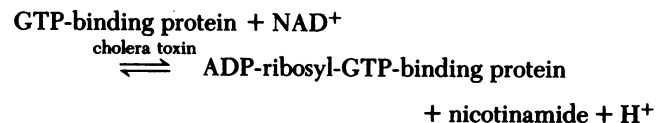
† In contrast to nucleotide stimulation, F⁻ activation is considerably less decreased after affinity chromatography. See also ref. 14.

GTP analog (GTPγS). On the basis of the ³²P incorporation, one estimates a 60-fold purification of the 42,000-M_r protein by the affinity chromatography.

Previously reported experiments have demonstrated the reversible dissociation of the soluble adenylate cyclase complex into two fractions upon chromatography on GTP-Sepharose. The fraction retained by the column contained GTP-binding proteins which were released by a GTP analog and had negligible adenylate cyclase activity. The fraction that was not retained contained an enzyme that had partially lost its response to guanyl nucleotides and F⁻. Reactivation occurred on recombination of the two fractions (14). To test which of the above fractions is modified by cholera toxin, the regulatory and catalytic fractions derived from Lubrol PX-solubilized adenylate cyclase preparations of toxin-treated and nontreated membranes were interchanged. As can be seen from Table 3, only the GTP-binding fraction obtained from the toxin-treated preparation was able to confer GTP stimulation on the catalytic component depleted of GTP-binding proteins, obtained from both the nontreated and the toxin-treated acceptor preparation. On the other hand, only the regulatory component prepared from normal membranes was able to restore F⁻ activation to depleted adenylate cyclase prepared from either nontreated or toxin-treated membranes. The GTP-binding fraction from cholera toxin-treated membranes did not confer F⁻ stimulation even when combined with depleted enzyme prepared from nontreated membranes. These findings extend previous observations (14), which indicated that F⁻ stimulation is conferred to adenylate cyclase *via* the guanyl nucleotide-binding proteins.

Reversal of Toxin Action by Nicotinamide. D. Shirisli and Z. Selinger (personal communication) found recently that the changes in adenylate cyclase activity brought about by cholera toxin treatment of turkey erythrocyte membranes can be partially reversed at low pH in the presence of toxin and nicotinamide. Using similar conditions, we found partial reversal of the toxin-induced enhancement of GTP activation and inhibition of F⁻ activation of pigeon erythrocyte membrane adenylate cyclase, which was accompanied by a reduction of the radioactivity in the 42,000-M_r protein (Table 4). This suggests strongly that cholera toxin catalyzes, as diphtheria toxin does

(8), a reversible ADP-ribosylation that is described by the following reaction:



An ADP-ribosylation reaction is also indicated by the finding that ADP-ribose (60%) and AMP (40%) were detected after acid treatment of the labeled 42,000-M_r protein (0.1 M HCl, 100°, 15 min). AMP is apparently formed upon cleavage of the pyrophosphate bond of ADP-ribose. Cholera toxin has recently been shown to have enzymatic activity and to catalyze the transfer of ADP-ribose to H₂O (24).

Table 4. Reversal of the cholera toxin effects by nicotinamide at pH 6.1

Additions to cholera toxin-treated membranes	Adenylate cyclase activity, pmol/mg per min			³² P in 42,000-M _r protein, pmol/mg protein
	GTP	isoproterenol	NaF	
None	25	110	45	3.8
Nicotinamide	28	111	46	3.7
Cholera toxin	31	120	41	4.0
Cholera toxin + nicotinamide	10*	41*	149*	1.7

Membranes were treated with toxin at 100 µg/ml plus 20 µM [³²P]-NAD⁺, pH 7.4, and then washed. Subsequently, the treated membranes (4 mg/ml) were mixed with an equal volume of cytosol containing 130 mM NaCl, 10 mM KCl, 2 mM MgCl₂, 1 mM ATP, 10 mM phosphoenolpyruvate, pyruvate kinase at 50 µg/ml, 1 mM dithiothreitol, and 0.1 M 2-(N-morpholino)ethanesulfonic acid (Mes) buffer, pH 6.1. Nicotinamide (50 mM) and toxin (180 µg/ml, preincubated with the cytosol) were added where indicated. After incubation for 60 min at 30°, the reaction was terminated by cooling in an ice bath. The membranes were washed with Mops/saline and assayed for adenylate cyclase activity in the presence of 0.1 mM GTP, 50 µM isoproterenol, or 8 mM NaF. ³²P-Labeled protein was analyzed as described in Fig. 1 and *Materials and Methods*.

* Adenylate cyclase activities of nontreated membranes that have been incubated at pH 6.1 were (pmol/mg per min): GTP, 2.5; isoproterenol + GTP, 17; NaF, 175.

The Role of Cytosolic Factors. With the adenylate cyclase system from pigeon erythrocyte membranes, no factor in addition to saturating concentrations of cholera toxin and NAD^+ was required for the modification of adenylate cyclase activity. However, at low NAD^+ concentrations the addition of cytosol, and to some extent also of ATP and an ATP-regenerating system, enhanced the toxin effects on adenylate cyclase activity 3- to 6-fold, and caused a proportional increase in the labeling of the 42,000- M_r protein. The cytosol itself was only barely labeled in the presence of [^{32}P]NAD $^+$ and cholera toxin and no radioactivity was found in a protein with M_r 42,000. Thus, the 42,000- M_r protein covalently modified by toxin treatment appears to be located exclusively in the membrane. The nature of the cytosolic factor(s) (25-28), the mechanism by which the factor enhances the cholera toxin activation of the adenylate cyclase, and the concomitant covalent modification of the GTP-binding protein require further study.

DISCUSSION

Previous study has shown that a 42,000- M_r protein that is specifically labeled by a photoreactive GTP analog comigrates with the adenylate cyclase in a sucrose density gradient (14, 16). The experiments with cholera toxin reported herein strongly indicate that the 42,000- M_r protein is indeed the GTP-binding component of the adenylate cyclase. Moreover, the cross-reconstitution experiment (Table 3) suggests that both the toxin-induced enhancement of GTP stimulation and the suppression of F^- stimulation are due to the covalent modification of a regulatory GTP-binding component with M_r of 42,000. Because the toxin-induced enhancement of adenylate cyclase stimulation by GTP was shown to be due to the inhibition of the hydrolysis of GTP at the regulatory site (9), it follows that the 42,000- M_r protein functions as a GTPase.

An interesting observation was that somewhat higher concentrations of toxin were required to suppress the F^- stimulation than those necessary to enhance the GTP stimulation of the adenylate cyclase (Fig. 2, ref. 9). This suggests that the toxin effect on F^- stimulation requires the modification of more than one site on the GTP-binding protein. An alternative explanation can be based on the assumption of two functionally different classes of GTP-binding proteins, one of which is involved in GTP stimulation and the other in F^- activation. Assuming a modification of one site on each 42,000- M_r molecule, it turns out that the amount of this protein in the pigeon erythrocyte is 8 pmol/mg of membrane protein. This amount is 4-8 times the amount of β -adrenergic receptor in the membrane (T. Pfeuffer, unpublished data). The significance of nonstoichiometric amounts of the adenylate cyclase components is as yet unknown.

The present findings indicate that cholera toxin catalyzes an ADP-ribosylation, and has thus an enzymic activity similar to that of diphtheria toxin (8). The analogy between the actions of the two toxins is even more striking because both of them modify a GTP-binding protein. It should therefore be considered that the GTP-binding component of the adenylate cyclase and the elongation factor 2 of eukaryotic protein biosynthesis might have segments with similar amino acid sequences.

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