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Biochemistry

Transfer of ADP-ribose from NAD to choleragen: A subunit acts as catalyst and acceptor protein

(adenylate cyclase)

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ABSTRACT **Choleragen selectively incorporates ^3H from [^3H]NAD labeled on the adenosine moiety and not ^{14}C from [^{14}C]NAD labeled on the nicotinamide moiety. This reaction does not require protein in addition to choleragen. Incorporation of isotope does not proceed at 4°, requires dithiothreitol, is stable after extensive washing with cold trichloroacetic acid, and is decreased 80% by boiling in trichloroacetic acid. Studies with the A and B subunits of choleragen show that the A subunit catalyzes ADP-ribosylation and serves as an acceptor protein. The B subunit does not show catalytic or acceptor activity. We conclude that choleragen and its A subunit catalyze the hydrolysis of NAD and the enzymatic transfer of ADP-ribose to the A subunit.**

Choleragen, the toxin of *Vibrio cholerae*, has been shown to activate adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] in a wide variety of intact mammalian cells (1). Gill first demonstrated that broken cell preparations unresponsive to choleragen require the addition of NAD to restore choleragen stimulation of adenylate cyclase (2), suggesting the involvement of NAD in the activation process. Activation of adenylate cyclase is a function of the choleragen A subunit (3) and of peptide fragments of the A subunit (4).

Choleragen B subunit binds specifically to the monosialoganglioside GM_1, facilitating the entry of the A subunit into the intact cell (5, 6). Recently, Moss and coworkers (7) demonstrated that choleragen, and specifically its A subunit, possess NAD-hydrolyzing activity (NAD^+ glycohydrolase; EC 3.2.2.5), and that the toxin is capable of ADP-ribosylating L-arginine (8). We report that the ADP-ribosyl moiety of NAD is enzymatically transferred to choleragen and specifically to the choleragen A subunit. Holotoxin and the A subunit both catalyze this enzymatic transfer and serve as acceptor proteins for ADP-ribosylation.

EXPERIMENTAL PROCEDURE

Materials. Choleragen was purchased from Schwarz-Mann. [*Adenine*-2,8-^3H]NAD (3.2 Ci/mmol) was obtained from New England Nuclear and [*carbonyl*-^{14}C]NAD (53 mCi/mmol) from Amersham/Searle. Sephadex G-75 was purchased from Pharmacia Fine Chemicals. Trypsin was obtained from Worthington Biochemical Corp. NAD and dithiothreitol were purchased from Calbiochem.

Assay for ADP-Ribosylation. The reaction mixture, unless otherwise stated, contained 200 mM potassium phosphate buffer, pH 7.0, 20 mM dithiothreitol, and the indicated amounts of protein added in the buffer supplied with the choleragen (50 mM Tris·HCl, pH 7.5/200 mM NaCl/1 mM EDTA/3 mM NaN_3) in a total volume of 0.1 ml.

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The reaction was initiated by the addition of NAD (2 mM final concentration containing 3 μCi of [*adenine*-2,8-^3H]NAD) and the incubation was continued at 37° for 2 hr. The reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid; the mixture was filtered on a Millipore type HA filter, pore size 45 μm. The filter was washed with five 3-ml portions of cold 5% trichloroacetic acid and placed in ACS (Amersham/Searle); the radioactivity was measured. ADP-ribose incorporation was calculated from incorporation of ^3H into trichloroacetic acid-insoluble material after incubation with [*adenine*-2,8-^3H]-NAD.

Separation of Choleragen into A and B Subunits. Choleragen (5 mg in 1 ml) was dialyzed against 6.5 M urea in 0.1 M glycine (pH 3.2) for 36 hr at 4° to allow dissociation of choleragen into its A and B subunits (7). The dissociated subunits were applied to a Sephadex G-75 column (1.2 × 80 cm) equilibrated with the dialysis buffer and eluted with the urea/glycine solution at a flow rate of 3 ml/hr. Fractions of 1.2 ml were collected. Those corresponding to the A subunit (fractions 32–34) and to the B subunit (fractions 43–46) were pooled and dialyzed extensively at 4° against the choleragen buffer.

The protein concentrations of the pooled subunit fractions were determined by the method of Lowry *et al.*, with bovine serum albumin as standard (9).

RESULTS

Table 1 shows that choleragen promoted the incorporation of ^3H from the adenosine moiety but not ^{14}C from the nicotinamide moiety of NAD into trichloroacetic acid-insoluble material. The incorporated ^3H was decreased by about 80% when the reaction mixture was boiled for 30 min in 5% trichloroacetic acid before it was filtered. Thus, the incorporation of ^3H from NAD into trichloroacetic acid-insoluble material catalyzed by choleragen has the characteristics of covalent incorporation of the ADP-ribose moiety from NAD into protein (10, 11). Bovine serum albumin did not selectively incorporate isotope from the adenosine or the nicotinamide moiety of NAD.

Based on a molecular weight of 83,000 for the choleragen holotoxin, about 0.77 pmol of ADP-ribose was incorporated per pmol of toxin. In contrast, only about 0.06 pmol of ADP-ribose was detected per pmol of bovine serum albumin (65,400 daltons). The ADP-ribose acceptor activity does not appear to be due to contaminating material associated with the choleragen preparation. Similar results were found for several batches of choleragen. Moreover, when choleragen was dissociated by prolonged dialysis against urea/glycine and the subunits were separated by gel filtration only two distinct protein peaks, corresponding to subunits A and B, were obtained (Fig. 1). A similar pattern was observed by Moss *et al.* with a Bio-Gel P-60 column (7).

Table 2 shows that [^3H]ADP-ribosylation of choleragen was

Table 1. Incorporation of label into trichloroacetic acid-insoluble material using [*adenine*-2,8-^{3}H]NAD and [*carbonyl*-^{14}C]NAD

Protein added	Acid-insoluble ^{3}H and ^{14}C, pmol	
	^{3}H	^{14}C
Choleragen	370	34
Choleragen*	82	18
Bovine serum albumin	39	45
Bovine serum albumin*	29	34

Assays were performed as described in *Experimental Procedure* except that the mixture contained 4 μCi of [*carbonyl*-^{14}C]NAD in addition to [*adenine*-2,8-^{3}H]NAD. Forty micrograms of either choleragen or bovine serum albumin were present in each assay. Values of trichloroacetic acid-insoluble ^{3}H and ^{14}C are the means of triplicate determinations.

* Reaction was stopped with 3 ml of 5% trichloroacetic acid. The tubes were placed in a boiling water bath for 30 min and cooled to room temperature. The reaction mixture was filtered, and radioactivity of the filters was determined.

dependent on the presence of dithiothreitol and did not proceed when Tris·HCl was substituted for potassium phosphate buffer. Heating completely blocked the incorporation of label into choleragen. Incubation at 2° for 2 hr resulted in no detectable incorporation of ^{3}H into protein (results not shown). Trypsin caused a concentration-dependent loss of incorporated label.

Table 3 shows that the A subunit of choleragen possessed the ADP-ribose incorporating activity. Doubling the concentration of the A subunit resulted in a 3.6-fold increase in ADP-ribose incorporation. The B subunit alone, up to 10.5 μg, showed no incorporation of label. Addition of B subunit to A subunit did not enhance the incorporation of ADP-ribose into protein above the level of A subunit alone. The increase of ADP-ribosylation of the A subunit after the concentration of the A subunit was doubled was not stoichiometric.

DISCUSSION

Our results provide a demonstration of ADP-ribosylation of a protein by choleragen. Choleragen alone selectively incorporates ^{3}H from [^{3}H]NAD labeled on the adenosine moiety and not ^{14}C from [^{14}C]NAD labeled on the nicotinamide moiety. Incorporation is not decreased after the sample is washed 20

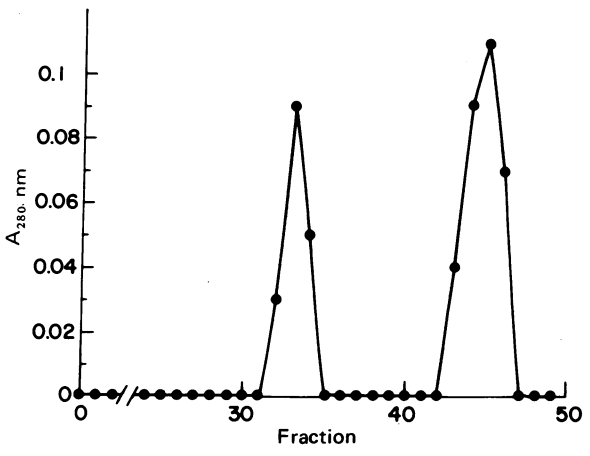

FIG. 1. Chromatography of dissociated choleragen on Sephadex G-75 column. Choleragen was dissociated by exposure to 6.5 M urea in 0.1 M glycine (pH 3.2), applied to the column, and eluted. Fractions 32–34 correspond to the choleragen A subunit and fractions 43–46 to the B subunit

Table 2. Some requirements for the ADP-ribosylation of choleragen

Reaction condition	[^{3}H]ADP-ribose incorporated, pmol
Complete	290
Minus dithiothreitol*	45
Minus potassium phosphate buffer (200 mM, pH 7.0) + Tris·HCl (200 mM, pH 7.0)	37
Minus toxin + boiled toxin†	33
Plus trypsin (μg/ml)‡	
450	183
220	211
45	238

Assay conditions are as described in *Experimental Procedure*. Each assay system contained 40 μg of choleragen. Values given are the means of three determinations.

* Water was substituted for the dithiothreitol.

† Choleragen was boiled at 100° for 10 min and 40 μg of the boiled protein was added to each assay system.

‡ After 2 hr of incubation in the complete reaction mixture, trypsin was added and incubation was continued for 30 min before it was stopped with trichloroacetic acid. Concentration given is the final concentration of trypsin.

times with 3 ml of 5% trichloroacetic acid (data not shown), but is decreased by about 80% by boiling in trichloroacetic acid.

The assay conditions that support choleragen-stimulated NAD hydrolysis (7) are similar to those that increase choleragen ADP-ribosylation. Choleragen-stimulated hydrolysis of NAD requires dithiothreitol, is stimulated by potassium phosphate, and does not proceed when Tris·HCl is substituted for potassium phosphate (7). Although the holotoxin supports ADP-ribosylation in our assay system, the requirement for a relatively high concentration of dithiothreitol (20 mM) suggests that dissociation of the holotoxin into its A and B subunits, and perhaps further dissociation of the A subunit, may be a prerequisite for choleragen ADP-ribosylation. Moss *et al.* (7) reported that heating increased choleragen-stimulated NADase activity. We found, however, that heating inhibits choleragen ADP-ribosylation.

ADP-ribosylation is catalyzed by the choleragen A subunit, and the A subunit is an acceptor protein for ADP-ribosylation. The B subunit shows neither catalytic nor acceptor activity. The A subunit (28,000 daltons) can be cleaved by sulfhydryl reagents into the A_1 (20,000–24,000 daltons) and A_2 (4000–7500 daltons) subunits (12). Matuo *et al.* (4) have shown that the A subunit,

Table 3. Activity of choleragen A and B subunits in catalyzing ADP-ribose incorporation into protein with NAD as donor

Protein added, μg	[^{3}H]ADP-ribose incorporated, pmol
Subunit A	
5.25	29
10.50	106
Subunit B	
5.25	0
10.50	1.5
Subunits A + B	
5.25 + 5.25	23

The subunits of choleragen were separated and the assay was performed as described in *Experimental Procedure*. Values given are the means of three determinations.

when incubated with sarcoma membranes, elaborates many small fragments and that fragments of the A subunit as small as 1400 daltons possess adenylate cyclase stimulating activity. We have not determined where on the A subunit ADP-ribosylation occurs.

In analogy to diphtheria toxin, the *in vitro* effects of choleragen require NAD and are mediated by the toxin A subunit (10, 11). Choleragen and diphtheria toxin catalyze NAD hydrolysis and ADP-ribosylation (7, 8, 10, 11). For diphtheria toxin, these are considered to be two separate reactions (11). The rate of hydrolysis of NAD by fragment A of diphtheria toxin has been calculated by Kandel and coworkers (11) to be six orders of magnitude lower than the rate of diphtheria toxin-catalyzed ADP-ribosylation of elongation factor II. They conclude it is unlikely that NAD glycohydrolase activity is a significant factor in diphtheria toxin toxicity (11). Gill has shown that the addition of ADP-ribose or nicotinamide to choleragen *in vitro* fails to activate adenylate cyclase in a system in which addition of choleragen plus NAD elevates adenylate cyclase over 12-fold. He concludes that NAD is not hydrolyzed to ADP-ribose and nicotinamide in choleragen activation of adenylate cyclase (2). If choleragen first hydrolyzed NAD to ADP-ribose and nicotinamide and later transferred ADP-ribose to an acceptor protein, the addition of ADP-ribose to choleragen in Gill's system should have resulted in cyclase activation, as he pointed out. It may be that choleragen-induced ADP-ribosylation is analogous to the primary reaction of diphtheria toxin, a group transfer reaction in which the nicotinamide moiety of NAD is replaced by an acceptor protein, in this case, the choleragen A subunit. Moss and Vaughan (8) tested a series of substances as acceptors for choleragen-catalyzed ADP-ribosylation and concluded that choleragen catalyzes the ADP-ribosylation of an arginine or related amino acid residue. In preliminary experiments with highly purified elongation factor I and elongation factor II (13), we found that these proteins were not substrates for choleragen-mediated ADP-ribosylation.

The data suggest one of two models for choleragen stimulation of adenylate cyclase. One is that stimulation results from the interaction of the ADP-ribosylated choleragen A subunit or some ADP-ribosylated fragment of the A subunit with the cell cyclase system. The second is that the choleragen A subunit simultaneously binds NAD and an acceptor protein and transfers ADP-ribose to the acceptor protein, resulting in an activation of the adenylate cyclase system.

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