

Activation of Adenylate Cyclase by Heat-Labile *Escherichia Coli* Enterotoxin

EVIDENCE FOR ADP-RIBOSYLTRANSFERASE ACTIVITY SIMILAR TO THAT OF CHOLERAGEN

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ABSTRACT Highly purified, polymyxin-released, low molecular weight *Escherichia coli* heat-labile enterotoxin (LT) catalyzed the hydrolysis of NAD to ADP-ribose and nicotinamide. This NAD glycohydrolase activity was stimulated by dithiothreitol and was independent of cellular components. Nicotinamide formation was enhanced by arginine methyl ester > D-arginine \cong L-arginine \cong guanidine. A 20-fold increase in activity was noted with arginine methyl ester, and maximal activity again required dithiothreitol. When the reaction was initiated with toxin, a delay was observed before a constant rate was established. The reaction products found after incubation of [adenine-U- 14 C]NAD and L-[3 H]arginine or unlabeled arginine methyl ester with the enterotoxin had mobilities on thin-layer chromatograms similar to the reaction products obtained after incubation of cholera toxin with these substrates and are consistent with the formation of ADP-ribose-L-arginine and ADP-ribose-L-arginine methyl ester, respectively. Both toxins, which catalyze the NAD-dependent activation of adenylate cyclase, thus appear to possess NAD glycohydrolase and ADP-ribosyltransferase activities. Although the activities of both toxins are dependent on dithiothreitol, *Escherichia coli* enterotoxin exhibited optimal activity in Tris(Cl $^-$) (pH 7.5) and was inhibited by high concentrations of potassium phosphate (pH 7.0) or low pH (sodium acetate, pH 6.2). It appears

that the optimal assay conditions as well as the kinetic constants for the reactants differ from those previously noted with cholera toxin. It is probable therefore that although the two toxins catalyze similar reactions, they differ in primary structure. The presence of transferase and glycohydrolase activities in structurally distinct toxins that activate adenylate cyclase strengthens our hypothesis that the ADP-ribosylation of arginine is a model for the NAD-dependent activation of adenylate cyclase; activation may result from ADP-ribosylation of the cyclase itself or of a protein that regulates its activity.

INTRODUCTION

Certain strains of *Escherichia coli* produce a heat-labile enterotoxin (LT),¹ which is believed to be responsible for the syndrome characteristic of "traveler's diarrhea" (1-5). The effects of the toxin are a result of the intracellular accumulation of cyclic AMP and activation of adenylate cyclase (6-13); Gill et al. (13), with a cell-free system derived from pigeon erythrocytes, have shown that the toxin activates adenylate cyclase in a process that is dependent upon NAD and ATP. Thus, the biochemical effects of the toxin are similar to those of cholera toxin (cholera toxin), an enterotoxin of *Vibrio cholerae*, which also causes the NAD-dependent activation of adenylate cyclase (14, 15). Although the mechanism of cholera toxin activation and NAD utilization has not been determined, it has been demonstrated (16-18) that both the holotoxin and its A protomer can catalyze the hydrolysis of NAD to ADP-

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¹ Abbreviation used in this paper: LT, *Escherichia coli* heat-labile enterotoxin.

ribose and nicotinamide (reaction [1]) and the transfer of the ADP-ribose moiety of NAD to arginine (reaction [2]). Because reaction (1) is analogous to that

NAD → ADP-ribose

+ nicotinamide + H⁺ (NAD glycohydrolase) (1)

NAD + arginine → ADP-ribose-L-arginine

+ nicotinamide + H⁺ (ADP-ribosyltransferase) (2)

catalyzed by diphtheria toxin (19), which inhibits protein synthesis as a result of the ADP-ribosylation of elongation factor II (20), and because cholera toxin catalyzes the ADP-ribosylation of an amino acid, it was proposed that cholera toxin activation of adenylate cyclase involves the ADP-ribosylation of the cyclase itself or of a regulatory protein (17). If cholera toxin and LT activate adenylate cyclase through similar mechanisms, and if the ADP-ribosylation of arginine in the presence of cholera toxin is, in fact, a model for the reaction critical to the activation process, then it would be presumed that LT should also catalyze reactions (1) and (2). We report here that a polymyxin-released LT of low mol wt ($\approx 20,000$) possesses both NAD glycohydrolase and ADP-ribosyltransferase activities. Furthermore, although LT and cholera toxin are apparently immunologically related (13, 21–27), they exhibit different requirements for the optimal expression of catalytic activity.

METHODS

NAD glycohydrolase and ADP-ribosyltransferase assays. NAD glycohydrolase and ADP-ribosyltransferase activities were assayed in a total volume of 150 μ l containing 2 mM [*carbonyl-¹⁴C]NAD ($\approx 40,000$ cpm), 20 mM dithiothreitol, and 50 mM potassium phosphate (pH 7.0) in the absence and presence of 75 mM arginine methyl ester, respectively. Substitutions were made in the assay as noted in legends for tables and figures. Assays were initiated with the addition of LT and incubated for 5 h at 30°C. Two 0.05-ml samples from each assay were then transferred to columns of AG 1-X2 (Bio-Rad Laboratories, Richmond, Calif.) from which [*carbonyl-¹⁴C]nicotinamide was eluted for radioassay (16). All assays were run in duplicate.**

Purification of LT. The polymyxin-released LT was purified by procedures described previously (28) plus an additional step involving gel filtration on Ultrogel AcA-44 (LKB Instruments, Inc., Rockville, Md.). This toxin, which was previously shown to be of low mol wt ($\approx 20,000$), exhibited on polyacrylamide gels one major band with biological activity.

Materials. The sources of the materials used in the assays of NAD glycohydrolase and ADP-ribosyltransferase and the specific activities of the radioactive substrates have been described (16–18). Protein was determined by the method of Lowry et al. (29).

RESULTS

The highly purified, polymyxin-released LT catalyzed the formation of [*carbonyl-¹⁴C]nicotinamide from*

TABLE I
Effect of Dithiothreitol and Arginine Methyl Ester on [*Carbonyl-¹⁴C]Nicotinamide Formation from [*Carbonyl-¹⁴C]NAD Catalyzed by LT**

Additions	Nicotinamide formed nmol/5 h
None	0.1
Dithiothreitol, 20 mM	1.7
Arginine methyl ester, 75 mM	2.1
Arginine methyl ester and dithiothreitol	28.8
Arginine methyl ester, dithiothreitol, and 400 mM potassium phosphate	12.4

Assays containing 2 mM [*carbonyl-¹⁴C]NAD (41,000 cpm) and 50 mM potassium phosphate, pH 7.0 (except as indicated), in a total volume of 0.15 ml were initiated with the addition of LT (107 μ g).*

NAD (Table I). The reaction was stimulated by dithiothreitol and arginine methyl ester and was inhibited by high concentrations of potassium phosphate (pH 7.0) (Table I). In assays containing potassium phosphate, arginine methyl ester, and dithiothreitol, and initiated with LT, there was a delay of at least 30 min before the maximal rate of nicotinamide production

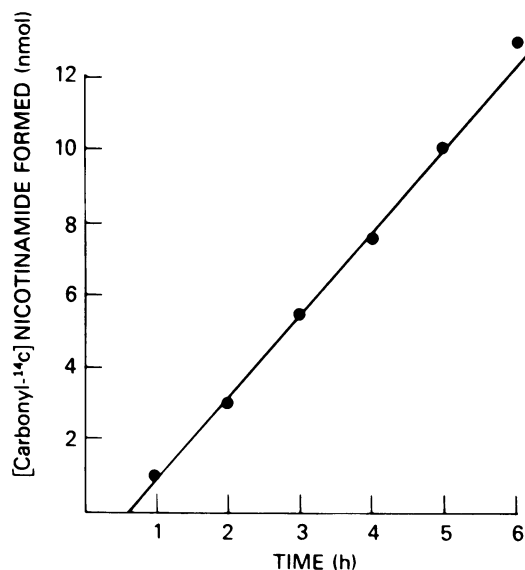


FIGURE 1 Formation of [*carbonyl-¹⁴C]nicotinamide from [*carbonyl-¹⁴C]NAD catalyzed by LT. Assays were initiated with the addition of LT (107 μ g) and contained 50 mM potassium phosphate, 20 mM dithiothreitol, 2 mM [*carbonyl-¹⁴C]NAD (2.2×10^5 cpm), and 75 mM arginine methyl ester in a total volume of 0.75 ml. At the indicated times, two 0.05-ml samples were transferred to columns of AG 1-X2 for separation of nicotinamide. Data are the means of values from duplicate incubations for [*carbonyl-¹⁴C]nicotinamide formation per 0.05-ml sample.****

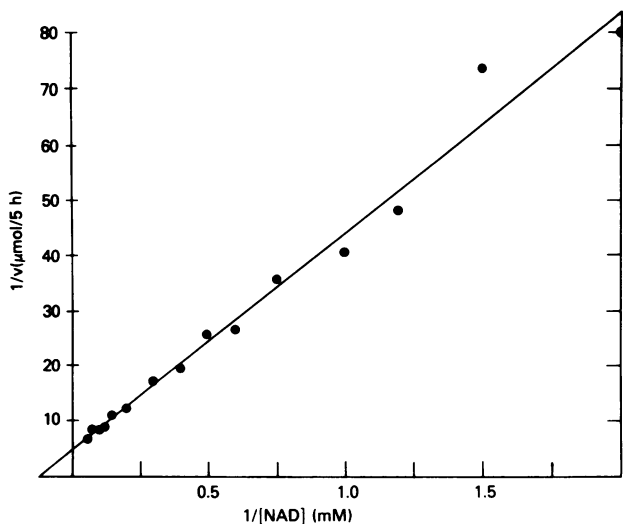


FIGURE 2 Determination of the K_m for NAD under the standard assay conditions for LT. Assays were carried out as described in Methods with 107 μg of LT and 75 mM arginine methyl ester except that the NAD concentration was varied as indicated. Each assay contained $\approx 42,300$ cpm of [carbonyl- ^{14}C]NAD.

was established; the rate was then constant for over 5 h (Fig. 1). As shown in Fig. 2, the K_m for NAD under these conditions was 8 mM. At pH 7.5 (Tris buffer) nicotinamide production in the presence of arginine methyl ester was greater than it was at pH 8 (Tris or glycine buffer) or at pH 7.0 (phosphate buffer). At pH 6.2 (acetate) nicotinamide production was only 10% of that at pH 7.5 (Table II).

In addition to arginine methyl ester, which increased nicotinamide formation 20-fold, L-arginine, D-arginine,

TABLE II
Effect of Buffers and pH on [Carbonyl- ^{14}C]Nicotinamide Formation from [Carbonyl- ^{14}C]NAD in the Presence of Arginine Methyl Ester Catalyzed by LT

Buffer (50 mM)	Nicotinamide formed nmol/5 h
Sodium acetate pH 6.2	5.1
Potassium phosphate pH 6.5	17.4
pH 7.0	40
Tris(Cl^-) pH 7.5	52
pH 8.0	35
Glycine (HCl) pH 8.0	41
pH 8.5	23

Assays were carried out as described in Methods with 107 μg of LT, 75 mM arginine methyl ester, and buffer as indicated.

TABLE III
Effect of Amino Acids and Related Compounds on Formation of [Carbonyl- ^{14}C]Nicotinamide from [Carbonyl- ^{14}C]NAD Catalyzed by LT

Additions (75 mM)	Nicotinamide formed nmol/5 h
None	1.9
Arginine methyl ester	40.0
Lysine	2.5
L-Arginine	17.0
D-Arginine	22.0
Guanidine	21.0
Histidine	2.1
Urea	2.1
Citrulline	1.5
Serine	2.0

Assays were carried out as described in Methods with 107 μg of LT and other additions as indicated.

and guanidine were also effective, although to a lesser degree, in stimulating the reaction (Table III). The K_a for both arginine methyl ester and arginine was 240 mM, but the V_{max} in the presence of arginine methyl ester was twice that with arginine (Fig. 3). Lysine, citrulline, histidine, and serine were ineffective (Table III).

Formation of ADP-ribose-L-arginine and ADP-ribose-L-arginine methyl ester. After incubation of [adenine- ^{14}C]NAD and L- ^3H]arginine or arginine methyl ester with cholera toxin, the reaction products

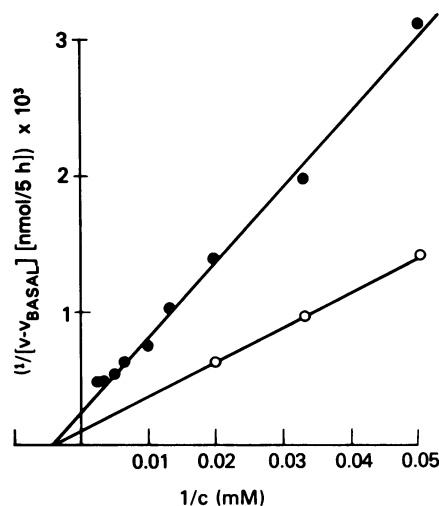


FIGURE 3 Determination of the K_a for arginine and arginine methyl ester. Assays with 37 μg of LT were carried out as described in Methods except that the concentration of NAD was 8 mM ($\approx 43,700$ cpm) and the concentrations (c) of arginine (●) or arginine methyl ester (○) were varied as indicated. V_{basal} = nmol of [carbonyl- ^{14}C]nicotinamide formed in the absence of arginine or arginine methyl ester = 155 nmol, i.e., less than 10% of the maximum observed with arginine.

can be separated from ADP-ribose, NAD and arginine, or arginine methyl ester by thin-layer chromatography with isobutyric acid:NH₄OH:H₂O, 66:1:33, on DEAE-cellulose or cellulose, respectively (17). After incubations with LT, reaction products with identical mobilities in both systems were observed. The compounds formed in the presence of either LT or cholera toxin contained ¹⁴C (and ³H, where L-[³H]arginine was present), reacted with ninhydrin and quenched the background fluorescence of plates viewed in ultraviolet light. These results are consistent with the formation of an ADP-ribose-L-arginine product catalyzed by both LT and cholera toxin. As expected, the formation of the ADP-ribose-L-arginine derivative was associated with the formation of [*carbonyl*-¹⁴C]nicotinamide as demonstrated in parallel experiments with [*carbonyl*-¹⁴C]NAD and unlabeled arginine or arginine methyl ester as substrates. Thus, as has been previously noted with cholera toxin (17), LT appears to be capable of transferring the ADP-ribosyl moiety of NAD to L-arginine to yield ADP-ribose-L-arginine with release of free nicotinamide and H⁺.

DISCUSSION

Several bacterial toxins require NAD for their effects on animal cells. The best investigated of these are diphtheria and *Pseudomonas* toxins, which catalyze the transfer of the ADP-ribose moiety of NAD to an acceptor protein, elongation factor II (20, 30). This protein is necessary for protein synthesis, and its ADP-ribosylation results in inhibition of the process. Like these toxins, both cholera toxin (an enterotoxin of *Vibrio cholerae*) and LT require NAD (13, 14); they, however, appear to exert their effects on cells by activating adenylate cyclase. Although the role of NAD in adenylate cyclase activation has not been defined, it has been shown that cholera toxin can catalyze the hydrolysis of NAD to ADP-ribose and nicotinamide (16–18), a reaction analogous to the abortive reaction catalyzed by both diphtheria and *Pseudomonas* toxins (19, 31). In addition, cholera toxin will catalyze the transfer of the ADP-ribose moiety of NAD to arginine (17). In view of the NAD requirement for cholera toxin action, as well as the facts that cholera toxin can hydrolyze NAD and transfer the ADP-ribose moiety of NAD to a basic amino acid, which could serve as a model for an acceptor amino acid in the acceptor protein, it was proposed that cholera toxin activation of adenylate cyclase proceeded by the ADP-ribosylation of the cyclase itself or a protein critical to cyclase activation. The protein acceptor for the ADP-ribose of NAD in the cholera toxin-catalyzed reaction has not been identified.

We have now shown that a heat-labile, low molecular weight toxin isolated from *E. coli*, which causes both the NAD-dependent activation of adenylate cyclase in

cell-free pigeon erythrocyte preparations (13) and accumulation of intracellular cyclic AMP (6, 7), possesses both NAD glycohydrolase and ADP-ribosyltransferase activities. The polymyxin-released LT thus appears to be functionally equivalent to cholera toxin. Other investigators have reported that antibodies to cholera toxin will cross-react with LT (13, 21–27). As noted here, however, the requirements for demonstration of optimal ADP-ribosyltransferase activity of LT differ considerably from those observed with cholera toxin. Whereas cholera toxin activity was enhanced over eight-fold by increasing the potassium phosphate concentration from 50 mM to 400 mM (16), LT was inhibited by more than 50%. Sodium acetate (pH 6.2) was as good a buffer as potassium phosphate for cholera toxin (16), although it inhibited LT activity by over 80%. Indeed, Tris(Cl⁻), pH 7.5, which was a poor buffer for cholera toxin gave excellent ADP-ribosyltransferase activity with LT. The *K_m* for NAD and the *K_a* for arginine were higher for LT than for cholera toxin. It appears, therefore, that although both toxins catalyze similar reactions, require thiol, exhibit a lag in reaction rate, and are immunologically similar, they may differ in structure. The fact that the ability to catalyze the transferase and glycohydrolase reactions is preserved in structurally distinct toxins which activate adenylate cyclase lends further support to the theory that the ADP-ribosylation of arginine, or similar amino acid, may be critical to toxin action. As proposed earlier (17), the transferase reaction would serve as a model for the ADP-ribosylation of the cyclase or of its regulatory protein.

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