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Biochemistry

Hydrolysis of nicotinamide adenine dinucleotide by choleragen and its A protomer: Possible role in the activation of adenylate cyclase

(gangliosides/ADP-ribosylation)

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ABSTRACT Choleragen and the isolated A protomer catalyzed the hydrolysis of NAD to ADP-ribose and nicotinamide. The protein with NADase activity (NAD nucleosidase; NAD glycohydrolase, EC 3.2.2.5) migrated on polyacrylamide gels with choleragen, and chromatographed on Bio-Gel P-60 columns with the A protomer. The NADase activity of choleragen and of the A protomer was increased markedly in acetate and phosphate buffers, and enhanced over 10-fold by dithiothreitol in high concentration. NAD hydrolysis was proportional to choleragen concentration; the Michaelis constant for NAD was about 4 mM with both choleragen and the A protomer. The demonstration that the A protomer of choleragen catalyzes an enzymatic reaction involving activation of the ribosyl-nicotinamide bond of NAD, a reaction analogous to those catalyzed by diphtheria toxin, supports the hypothesis that activation of adenylate cyclase by choleragen involves the ADP-ribosylation of an appropriate acceptor protein.

Choleragen is believed to exert its effects on mammalian cells through activation of adenylate cyclase (1). The initial step in the activation process is presumed to be the binding of the B protomer of choleragen to cell surface receptors, presumably the monosialoganglioside G_{M1} (2–7). The A protomer is then thought to penetrate the membrane and activate the cyclase (8–11). In cell homogenates, where the binding step can apparently be bypassed, the A protomer alone can activate adenylate cyclase in an NAD-dependent reaction (12–14). The role of NAD has not been defined, however, and some workers have not found an NAD requirement for cyclase activation (10). Gill (12) and Bitensky and coworkers (14) feel that choleragen catalyzes an enzymatic activation of adenylate cyclase; Cuatrecasas and coworkers believe that activation involves the direct binding of choleragen to the cyclase (10, 15).

We report here that choleragen and its A protomer catalyze the hydrolysis of NAD to ADP-ribose and nicotinamide (NAD nucleosidase activity; NAD glycohydrolase, EC 3.2.2.5). Although this probably represents an abortive reaction, it is analogous to the reactions catalyzed by diphtheria toxin, which causes inhibition of protein synthesis as a result of the NAD-dependent ADP-ribosylation of elongation factor II (16). The active Fragment A of diphtheria toxin exhibits a similar NADase activity (17). We propose, therefore, that choleragen activates adenylate cyclase through an NAD-dependent enzymatic reaction, which probably involves the ADP-ribosylation of the protein.

EXPERIMENTAL PROCEDURE

NADase Assay. The reaction mixture contained potassium phosphate buffer (pH 7.0), dithiothreitol, and [*carbonyl*-^{14}C]NAD at the concentrations indicated in the table and figure legends. Assays were initiated by the addition of choleragen in 50 mM Tris·HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 3 mM NaN_3. The purified A or B protomers were dialyzed against the same buffer prior to assay. Following incubation at 37° for the time specified in the legends, 0.1 ml samples were applied to Dowex-1 columns (0.5 × 4 cm) that were prepared as described below, and washed with 2 ml of 20 mM Tris·HCl, pH 7.5, prior to use. The [*carbonyl*-^{14}C]nicotinamide was eluted with five 1-ml portions of 20 mM Tris·HCl, pH 7.5. The effectiveness of the Dowex-1 separation for the quantitative isolation of [*carbonyl*-^{14}C]nicotinamide was confirmed by thin-layer chromatography (Table 1). Of added [*carbonyl*-^{14}C]nicotinamide, 97% was recovered following Dowex-1 chromatography over a wide range of nicotinamide concentrations (0.17 μM to 1.25 mM).

Materials. Bio-Gel P-60 was obtained from Bio-Rad Laboratories, and equilibrated with 6.5 M urea, 0.1 M glycine, pH 3.2. Dowex AG 1-X2 (Bio-Rad), 100–200 mesh in the chloride form, was washed with 0.5 M NaOH, water until neutral, 0.5 M HCl, and water until neutral before the final washing with Tris buffer described above. Protein was determined by the method of Lowry *et al.* (18). Choleragen was obtained from Schwarz/Mann. [*carbonyl*-^{14}C]Nicotinamide adenine dinucleotide (50 mCi/mmol) and nicotinamide [*U*-^{14}C]adenine dinucleotide (280 mCi/mmol) were purchased from Amersham/Searle. Dithiothreitol was obtained from Calbiochem and Schwarz/Mann, NAD from Sigma, and cellulose thin-layer plates from E. M. Laboratories.

Polyacrylamide gels were run in 36 mM Tris, 30 mM sodium phosphate, 1 mM EDTA, pH 7.55, for 20 min at 1 mA per tube, and then 2.5 hr at 6 mA per tube. Triphenylamine and *N,N'*-methylene-bis-acrylamide were purchased from Eastman, (*N,N,N',N'*-tetramethylethylenediamine (TEMED) and ammonium persulfate from Bio-Rad.

RESULTS

As shown in Table 1, choleragen catalyzed the hydrolysis of [*carbonyl*-^{14}C]nicotinamide adenine dinucleotide and nicotinamide [*U*-^{14}C]adenine dinucleotide to [*carbonyl*-^{14}C]nicotinamide and [*U*-^{14}C]ADP-ribose, respectively. These reaction products were identified by their migration with authentic compounds on thin-layer chromatograms (Table 1).

The NADase activity migrated with choleragen on polyacrylamide gels (Fig. 1). After dissociation of choleragen in 6.5 M urea, 0.1 M glycine at pH 3.2 (19), and separation of the $A(A_1,A_2)$ and B protomers by chromatography on a Bio-Gel P-60 column, the NADase activity was recovered with the A protomer (Fig. 2).

As shown in Fig. 3, hydrolysis of [*carbonyl*-^{14}C]NAD was directly proportional to choleragen concentration. The NADase activity of choleragen and of the isolated A protomer was markedly enhanced by relatively high concentrations of dithiothreitol (Table 2). As shown in Table 3, when the NADase activities of choleragen and of the A protomer were assessed in 200 mM buffers of different composition and pH in the

Table 1. Identification of the products of NAD hydrolysis by choleragen as nicotinamide and ADP-ribose

Exp.	Choleragen	Solvent	^{14}C applied, cpm	^{14}C recovered after chromatography: Total, cpm	Percentage of total cpm: NAD	Nicotinamide	ADP-ribose
A	None	I	1110	1080	90	5.5	
		II	1110	1090	92	4.7	
		Assay*	1110	—	—	4.4	
	100 μg	I	1130	1110	48	46	
		II	1130	1120	49	45	
		Assay*	1130	—	—	46	
B	None	I	1160	1170	84		7.4
	100 μg	I	1180	1120	53		40

Reaction mixtures contained 2 mM NAD, 20 mM dithiothreitol, and 200 mM potassium phosphate, pH 7.0, in a total volume of 0.2 ml plus [*carbonyl*-^{14}C]NAD in Exp. A and [*U*-^{14}C]adenine-NAD in Exp. B. After addition of 0.02 ml of a solution of choleragen (5 mg/ml) or diluent, and incubation for 2 hr at 37°, 0.05 ml samples were diluted with 0.45 ml of water. Samples (0.01 ml) of the diluted mix were taken for assay of the total ^{14}C and for application to cellulose plates. Standard samples of NAD, nicotinamide, and ADP-ribose were also applied and the thin-layer chromatograms developed with isobutyric acid:NH_4OH:water, 66:1:33 (Solvent I) or ethanol:1 M ammonium acetate (pH 7.5), 7:3 (Solvent II) for 5 hr. Each sample lane was divided into 1 cm segments (15 total) from which the cellulose was scraped for radioassay.

* Triplicate samples (0.01 ml) were applied to columns of Dowex-1 and eluted as described in *Experimental Procedure*. Mean recovery of ^{14}C in nicotinamide eluate is expressed as percentage of that applied to column.

presence of 20 mM dithiothreitol, only in the acetate and phosphate buffers was there appreciable activity. Increasing the concentration of the potassium phosphate buffer to 400 mM more than doubled the amount of NAD hydrolyzed. As shown in Fig. 4, the Michaelis constant for NAD was 3.8 mM with both choleragen and the purified A protomer.

DISCUSSION

Although it is well known that choleragen activates adenylate cyclases from many sources, the intermediate steps in this process have not been delineated. The initial event appears to involve binding of choleragen through its B protomer to a cell surface receptor, the monosialoganglioside G_{M1} (2–7, 20). The subsequent steps are less well defined. Gill (12) has shown that choleragen in the presence of NAD will activate adenylate cyclases in cell-free systems. Under these conditions the isolated A_1 subunit of choleragen is effective and activation does not require the B subunit or the G_{M1} surface receptor (8). Bennett *et al.* (15) have suggested that activation results from the direct

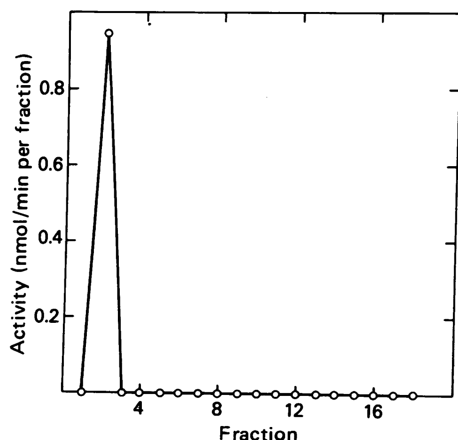

FIG. 1. Comigration of choleragen and NADase activity on polyacrylamide gels. Choleragen (125 μg in 0.025 ml) was applied to each of four 7.5% polyacrylamide gels. After electrophoresis two gels were stained for protein. The other two gels were sliced into 5 mm segments which were eluted for 14 hr at room temperature with 0.4 ml of the buffer in which the choleragen solutions were prepared. Assay reactants were added to the tubes containing the gels and elution buffer, bringing the final volume to 0.6 ml, containing 2 mM [*carbonyl*-^{14}C]NAD (50,000 cpm), 200 mM potassium phosphate, pH 7.0, and 20 mM dithiothreitol. After incubation for 2 hr at 37°, duplicate 0.2 ml samples were applied to Dowex-1 columns and eluted as described in *Experimental Procedure*. 57% of the applied NADase activity was recovered from the gels. The stained protein band was in fraction 2.

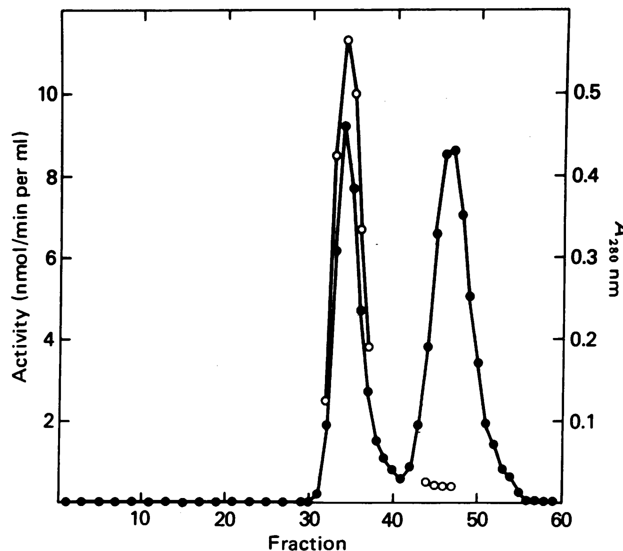

FIG. 2. Cochromatography of NADase activity and the A protomer of choleragen. Choleragen (5 mg in 1 ml) was dialyzed against 6.5 M urea in 0.1 M glycine, pH 3.2, for 36 hr at 4° and then applied to a Bio-Gel P-60 column (1.2 × 88 cm) equilibrated with the urea-glycine solution. The A and B protomers of choleragen were eluted with the same solution; 0.92 ml fractions were collected and $A_{280\ nm}$ was determined (●). Fractions 32 to 37 and 45 to 48, corresponding to the A and B protomers, respectively (19), were dialyzed separately against 2 liters of the choleragen buffer for 24 hr. Samples (0.2 ml) of each fraction were assayed in a total volume of 0.3 ml containing 2 mM [*carbonyl*-^{14}C]NAD (33,800 cpm), 20 mM dithiothreitol, and 200 mM potassium phosphate, pH 7.0. After incubation for 2 hr at 37°, 0.1 ml samples were applied in duplicate to Dowex-1 columns and eluted as described in *Experimental Procedure*. NADase activity (○) is expressed as nmol [*carbonyl*-^{14}C]nicotinamide formed per min/ml of each fraction.

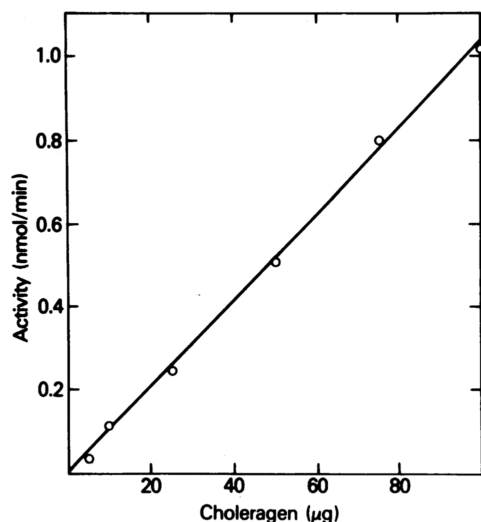

FIG. 3. NADase activity as a function of choleragen concentration. Assays were carried out in a total volume of 0.2 ml containing 2 mM [*carbonyl*-^{14}C]NAD (19,400 cpm), 200 mM potassium phosphate at pH 7.0, 20 mM dithiothreitol, and the indicated amounts of choleragen (in 0.05 ml of the choleragen buffer). After incubation for 1 hr at 37°, 0.1 ml samples were transferred to Dowex-1 columns which were eluted as described in *Experimental Procedure*.

interaction of choleragen with cyclase, whereas Gill (13) has concluded that an enzymatic process is involved.

We have now demonstrated that choleragen, and specifically the A protomer (consisting of A_1 and A_2 subunits), catalyzes the hydrolysis of NAD to nicotinamide and ADP-ribose. Diphtheria toxin, which also exhibits NADase activity (17), causes inhibition of protein synthesis in susceptible cells as a result of the reversible ADP-ribosylation of elongation factor II (16). In cell-free systems, ADP-ribosylation of elongation factor II by the toxin is accelerated by conditions that promote the liberation of the enzymatically active Fragment A from the other subunit (17). The latter, like the B subunit of choleragen, is believed to be responsible for the initial interaction of the diphtheria toxin with the cell surface receptors (21). The NADase activity of choleragen was enhanced by boiling (data not shown) and by incubation with dithiothreitol, treatments known to cause dissociation of the A and B protomers. Sulfhydryl-containing compounds in high concentration also facilitate the further dissociation of the A protomer into subunits A_1, which activates adenylate cyclase (8), and A_2. The dramatic effects of dithiothreitol (and of phosphate or acetate in high concentrations) on the NADase activity of choleragen and its A protomer could be explained in this way. Alternatively, these agents may directly influence the activity of the catalytic subunit.

Table 2. Dithiothreitol enhances NADase activity of choleragen and A protomer

Dithiothreitol (mM)	NADase activity (nmol/min per ml enzyme)	
	Choleragen	A protomer
0	0.3	0.0
2	0.4	2.1
20	3.8	7.3
50	4.9	7.7

Reaction mixtures contained 2 mM [*carbonyl*-^{14}C]NAD (34,100 cpm), 200 mM potassium phosphate at pH 7.0, and dithiotreitol as indicated in a total volume of 0.3 ml. After addition of choleragen (20 μg in 0.04 ml) or A protomer (0.04 ml of a solution of $A_{280\text{ nm}}$ = 0.691) and incubation at 37° for 2 hr, duplicate 0.1 ml samples were transferred to Dowex-1 columns and eluted as described in *Experimental Procedure*.

Table 3. Effect of buffer on NADase activity of choleragen and A protomer

Buffer	NADase activity (nmol/min per ml enzyme)	
	Choleragen	A protomer
None	0.0	0.0
Sodium acetate, pH 6.2, 200 mM	3.5	7.8
Potassium phosphate, pH 7.0,		
50 mM	1.3	2.9
200 mM	3.8	7.3
400 mM	10.5	16.4
Tris-Cl, pH 8.0, 200 mM	0.2	0.2
Glycine-Cl, pH 8.0, 200 mM	0.0	0.4
pH 8.5, 200 mM	0.0	0.1
Hydrazine-Cl, pH 9.5, 200 mM	0.0	0.2

Assays were carried out as described in Table 2 except that the concentration of dithiothreitol was 20 mM, and the buffer composition and concentration were varied as indicated.

The NADase activity of the A protomer of choleragen is apparently analogous to that exhibited by Fragment A of diphtheria toxin (17). It represents an abortive reaction in which water rather than a specific second substrate serves as an acceptor for the ADP-ribosyl moiety of NAD. It appears, therefore, that both choleragen and diphtheria toxin are capable of activating the ribosyl-nicotinamide bond of NAD. In the case of diphtheria toxin, the activated ADP-ribosyl moiety can be transferred to the appropriate acceptor protein, which has been identified as elongation factor II (16). Although ADP-ribosylation by choleragen has not been demonstrated, all of our observations are consistent with the conclusion that this is the mechanism by which the A protomer activates adenylate cyclase. It appears that choleragen may prove even more useful

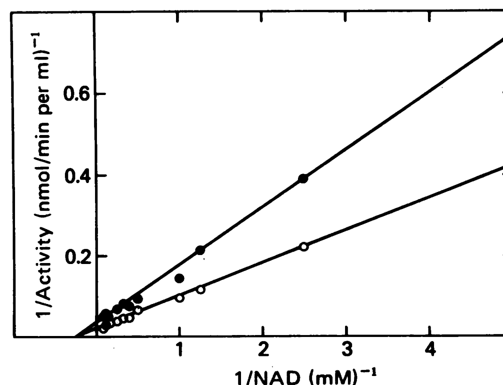

FIG. 4. Determination of the Michaelis constants for NAD hydrolysis by choleragen and the A protomer. Choleragen (0.04 ml) (0.5 mg/ml) or A protomer (solution of $A_{280\text{ nm}}$ = 0.691) was added to [*carbonyl*-^{14}C]NAD (31,100 cpm) (varied as indicated), 400 mM potassium phosphate at pH 7.0, and 20 mM dithiothreitol in a total volume of 0.3 ml. After incubation for 2 hr at 37°, duplicate 0.1 ml samples were transferred to Dowex-1 columns which were eluted as described in *Experimental Procedure*. NADase activity is expressed as nmol [*carbonyl*-^{14}C]nicotinamide formed per min/ml of choleragen (●) or A protomer (○).

than previously supposed in probing the nature of adenylate cyclase and its regulatory properties.

Note Added in Proof. We have recently demonstrated that choleragen, in the presence of NAD, catalyzes the ADP-ribosylation of arginine.

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