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The Adenylate Cyclase-Activating Activity of Cholera Toxin is not Associated with a Nicotinamide–Adenine Dinucleotide Glycohydrolase Activity

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The NAD^+ glycohydrolase activity of cholera-toxin samples can be separated from their adenylate cyclase-activating activity by polyacrylamide-gel electrophoresis and is inhibited by sodium dodecyl sulphate (which does not inhibit the action of toxin on cells), but not by antibodies to pure toxin. It is therefore probably not a true property of the toxin.

Cholera toxin is a potent activator of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] in a wide variety of eukaryotic cell membrane systems (for general review, see van Heyningen, 1977*a*). The activation requires NAD^+, ATP, a thiol reducing agent, and an unidentified factor from the cell sap, which is probably a protein (Gill, 1976). Recent evidence also indicates a requirement for GTP (Cassel & Selinger, 1977). There have been several reports that purified cholera toxin has an NAD^+ glycohydrolase activity (Moss *et al.*, 1976, 1977) and evidence that the toxin can catalyse an ADP-ribosylation with arginine as acceptor has been presented (Moss & Vaughan, 1977). There is also evidence for self ADP-ribosylation of cholera toxin (Trepel *et al.*, 1977). It has therefore been suggested that cholera toxin, in an analogous way to diphtheria toxin, may act in catalysing an ADP-ribosylation perhaps of adenylate cyclase itself, or at least of some component of the cyclase complex, thus leading to the activation of this enzyme.

This paper provides evidence that the simple NAD^+ glycohydrolase activity is not associated with the activation of adenylate cyclase, and so may well be due to contaminating protein.

Experimental

Purification of cholera toxin

Cholera toxin prepared from crude culture filtrates of *Vibrio cholerae*, kindly given by Dr. R. O. Thomson of the Wellcome Laboratories, Beckenham, U.K. (batch number VT 2214E), was purified as follows. All operations were carried out at 4°C, and all buffers were made 0.1 mM in phenylmethanesulphonyl fluoride immediately before use. The crude solution was made up to 30% of saturation (measured at 25°C) in $(NH_4)_2SO_4$, the precipitate discarded, and the supernatant made up to 60% of saturation in $(NH_4)_2SO_4$. The precipitate was dissolved in water, dialysed against 50mM-Tris/HCl, pH 7.4, and applied to a column (2cm diameter × 26cm) of DEAE-cellulose (Whatman DE-52) previously equilibrated with the same buffer. Material reacting with cholera antitoxin (van Heyningen, 1976*a*) was eluted with 200ml of a linear gradient of 0 to 0.1M-NaCl in the buffer, freeze-dried, dissolved in water, dialysed against 0.1M-Tris/HCl/0.1M-NaCl, pH 7.4, and applied to a column (2cm diameter × 92cm) of Ultrogel AcA-44 (LKB, South Croydon, Surrey, U.K.). The antigenic product was homogeneous as judged by the visible bands present on polyacrylamide gels in the presence and absence of sodium dodecyl sulphate and migrated with the same mobility as a sample of toxin purified by the method of Finkelstein & LoSpalluto (1970) given to us by the National Institutes of Health, Bethesda, MD, U.S.A., for whom it was prepared under contract.

Polyacrylamide gels

Polyacrylamide gels at pH 8.3 were run by the method of Davis (1964), without a stacking or sample gel. Crude cholera toxin (150μg) and purified toxin (50μg) were applied to gels in duplicate. One of the duplicate gels was stained for protein with Coomassie Brilliant Blue R250, and the other was sliced into 2mm segments which were eluted for 24h with shaking at 25°C in about 0.4ml of water. The gel slices were removed, the eluates were freeze-dried, and the residues were taken up in 30μl of water. Then 5μl of each eluate was taken for NAD^+ glycohydrolase assay, 10μl for adenylate cyclase-activating assay, and 5μl for immunodiffusion against rabbit antitoxin (van Heyningen, 1976*a*).

NAD^+ glycohydrolase assay

NAD^+ glycohydrolase activities were determined by the addition of 5μl of each gel-slice eluate to 10μl of an assay medium containing 0.2M-sodium acetate/0.02M-dithiothreitol/2mM-NAD^+ and 10nCi of [*carbonyl*-^{14}C]NAD^+ of specific radioactivity 53Ci/mol (The Radiochemical Centre, Amersham, Bucks., U.K.), pH6.2. Incubations were conducted at 37°C for 60min, after which time 10μl samples from each assay were spotted on 20cm×2cm strips of DE-81 chromatographic paper (Whatman, Maidstone, Kent, U.K.), and [^{14}C]nicotinamide was separated as previously described (Apps & Nairn, 1977), for the separation of NAD^+ and NADP. Under these conditions, nicotinamide migrated with the solvent front, and NAD^+ had an R_F of about 0.4.

Adenylate cyclase assay

The source of adenylate cyclase was a crude rat liver homogenate prepared by the method of van Heyningen (1976*b*). The crude particulate adenylate cyclase preparation was then made 2.5mM in NAD^+, ATP, dithiothreitol and isonicotinic acid hydrazide, an inhibitor of endogenous rat liver NAD^+ glycohydrolase that had no effect on the NAD^+ glycohydrolases of *V. cholerae*.

A 10μl sample of each gel-slice eluate was added to 15μl of crude particulate adenylate cyclase and incubated at 37°C. After 20min, 15μl of each preincubation mixture was transferred to tubes containing 10μl of assay medium. Each assay (final volume, 25μl) contained 20mM-Tris/HCl/5mM-$MgCl_2$/5mM-KCl/5mM-phosphoenolpyruvate/1mM-dithiothreitol/0.5mM-cyclic AMP/0.5mM-isobutylmethylxanthine/0.58mM-ATP/80μg of pyruvate kinase/ml/80μg of adenylate kinase/ml and 0.6μCi of [8-^{3}H]ATP (The Radiochemical Centre; specific radioactivity 23Ci/mmol). The assay medium was adjusted to pH8.0 with 1M-NaOH by using Phenol Red as internal indicator, before use.

Incubations were conducted at 37°C for 20min and reactions were terminated by the addition of 10μl of 25% trichloroacetic acid. Precipitated protein was removed by centrifugation and 10μl samples of each clear supernatant were spotted on 20cm×50cm glass plates spread to a layer thickness of 0.3mm with cellulose microcrystalline (Merck; obtained from BDH Chemicals, Poole, Dorset, U.K.), impregnated with a 1% solution of poly(ethyleneimine) (BDH Chemicals), and cyclic [^{3}H]AMP was separated by the method of Keirns *et al.* (1974) Cyclic [*adenine*-U-^{14}C]-AMP (5nCi) (The Radiochemical Centre; specific radioactivity 287Ci/mol) was spotted along with each sample to act as a check on individual plate recoveries. Cellulose scrapings were placed in 10ml of a Triton X-100/xylene-based scintillation cocktail (Fricke, 1975), 2ml of 25mM-$MgSO_4$ was added to solubilize the cyclic AMP from the cellulose particles, and the samples were counted for radioactivity in a Searle MK III liquid-scintillation spectrometer.

Results and Discussion

During our routine purification of cholera toxin from concentrated culture filtrates of *V. cholerae*, NAD^+ glycohydrolase activity was consistently found to decrease in fractions containing toxin and increase in fractions not containing toxin after each step in the purification procedure. In particular those fractions eluted from a DEAE-cellulose column containing no material precipitating with antibodies to pure cholera toxin were found to have about 90–99% of all the NAD^+ glycohydrolase activity eluted from the column. This immediately suggested that the NAD^+ glycohydrolase was a property of proteins other than the toxin. To test this hypothesis several experiments were done.

Treatment of purified cholera toxin with sodium dodecyl sulphate, a procedure known to increase the activation of adenylate cyclase by toxin (Gill, 1976; van Heyningen, 1977*b*), brought about a loss of most of the NAD^+ glycohydrolase activity in several different experiments. An identical treatment of the NAD^+ glycohydrolase eluted from DEAE-cellulose as described above caused a loss of nearly all the activity (Table 1).

Antibody prepared in rabbits against a different sample of cholera toxin purified by the method of Finkelstein & LoSpalluto (1970) (see van Heyningen, 1976*a*) inhibits the adenylate cyclase-activating activity of crude and purified toxin in cells. However, it had no effect on the NAD^+ glycohydrolase activity (Table 1).

Table 1. *Effect of sodium dodecyl sulphate and cholera antitoxin on the NAD^+ glycohydrolase activity of toxin and non-toxin preparations*

The results are the means of three experiments.

Preparation	NAD^+ glycohydrolase activity (nmol of NAD^+ hydrolysed/min per mg of protein)
Purified toxin	8.9
Purified toxin preincubated with rabbit antitoxin	9.5
Purified toxin preincubated with 0.1% sodium dodecyl sulphate for 10min at 37°C	2.7
Non-toxin protein eluted from DEAE-cellulose	5400
Same non-toxin protein preincubated with 0.1% sodium dodecyl sulphate for 10min at 37°C	47

It is also possible to separate the two activities by polyacrylamide-gel electrophoresis. Figs. 1(*a*) and 1(*b*) show the activity of NAD^+ glycohydrolase and adenylate cyclase-activating activities eluted from slices of gels of purified and crude toxin respectively. Activities are plotted against the mobility of the mid-point of each gel slice. In the pure-toxin gel, cholera toxin was detected by Ouchterlony immunodiffusion with mobilities 0.02–0.18, and in the crude-toxin gel (where cholera toxin constitutes only about 2% of the total protein) the toxin was detected with mobilities 0.09–0.20. In both gels at these mobilities there is, as expected, a strong peak of adenylate cyclase-activating activity. However, also in both cases, the NAD^+ glycohydrolase activity at these mobilities is virtually zero.

The very powerful NAD^+ glycohydrolase activity in crude culture filtrates of *V. cholerae* runs at mobility 0.5–0.6. The corresponding band on a gel stained for protein was very faint. Specific enzyme activities could not be calculated owing to the small amounts of material in each gel-slice eluate, but in a separate experiment the $V_{max.}$ of this NAD^+ glycohydrolase (partially purified) was found to be in the region of 3000–4000 nmol of NAD^+/min per mg of protein. In the pure-toxin gel in which only one band was visible on protein staining, that of cholera toxin, some of this NAD^+ glycohydrolase activity has persisted (mobility 0.5–0.6 in Fig. 1*a*). Thus although the purified material gives only one band on protein staining with a characteristic mobility for cholera toxin, the toxin is evidently not pure, and contains a significant amount of contaminating NAD^+ glycohydrolase activity.

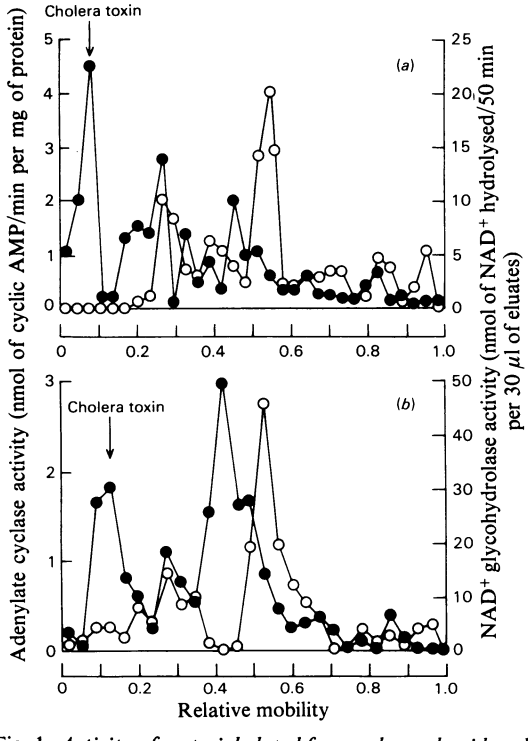

Fig. 1. *Activity of material eluted from polyacrylamide gels*
Purified (*a*) or crude (*b*) cholera toxin was subjected to polyacrylamide-gel electrophoresis at pH 8.3. Slices from each gel were immersed in water and the eluted protein was assayed for NAD^+ glycohydrolase activity (○) and for activation of adenylate cyclase in rat liver membranes (●) as described in the text.

A similar gel of cholera toxin, prepared by the method of Finkelstein & LoSpalluto (1970), gave similar results showing more than one band of adenylate cyclase-activating activity, but in this material the NAD^+ glycohydrolase activity was lower.

The peak of adenylate cyclase-activating activity at mobility about 0.4 in both gels may be due to free subunit A of the toxin. A similar gel of subunit A alone (purified by the method of van Heyningen, 1974) gave one band running in this position. Evidently the majority of this material has been removed from the toxin during purification. The other minor peaks of adenylate cyclase-activating activity, particularly on the gel of the purified toxin, may be due to small proteolytic fragments of cholera toxin, which are still capable of activating adenylate cyclase. Such fragments have been observed previously (Matuo *et al.*, 1976).

In neither of the two gels does the position of cholera toxin, nor the putative position of free subunit A, coincide with any appreciable NAD^+ glycohydrolase activity. Rather, this latter is present only as a contaminant clearly separable from the peaks of adenylate cyclase-activating activity.

One possible reason for differences between our results and those of other workers (Moss *et al.*, 1976, 1977; Moss & Vaughan, 1977), may be that their experiments were done in 0.2–0.4 M-phosphate buffer at pH 7.0. At this pH, even small concentrations of phosphate are known to catalyse the non-specific breakdown of NAD^+ to ADP-ribose and nicotinamide (Colowick *et al.*, 1951). In our experiments the use of such phosphate buffers resulted in as much NAD^+ hydrolysis whether cholera toxin was present in the assay mixtures or not. Acetate buffer at pH 6.2, which does not lead to extensive NAD^+ breakdown, was therefore used in the experiments described here.

Although NAD^+ is a strict requirement for adenylate cyclase activation by cholera toxin, the results presented in this paper do not support the hypothesis that cholera toxin (and specifically subunit A) acts as an enzyme directly catalysing the transfer of ADP-ribose at least to water. It remains possible that

the ADP-ribosylation of some other acceptor is part of the normal mechanism of the toxin.

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