The ADP-ribosyltransferase activity of cholera toxin and Escherichia coli heat-labile toxin

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Cholera toxin

CT, an enterotoxin produced by Vibrio cholerae, is responsible for the clinical manifestation of cholera which is characterized by severe fluid loss from the small intestine. The toxin acts by irreversibly stimulating adenylate cyclase in the basolateral membrane of the intestinal cell to raise cyclic AMP levels (Kimberg et al., 1971; Sharp & Hynie, 1971).

CT is a 84000-M, protein composed of two types of polypeptide subunits, A and B (Finkelstein, 1973; Lonroth & Holmgren, 1973; van Heyningen, 1974). The A subunit consists of two non-identical polypeptide chains, A1 and A2, linked by a single disulphide bridge. A1 is responsible for the activation of adenylate cyclase and can do this in cell-free systems independent of the B subunit (Gill & King, 1975; Wodnar-Filipowicz & Lai, 1976; Moss et al., 1979b).

The B subunit is believed to contain five identical polypeptide chains (Gill, 1976; Lai et al., 1977) and binds the toxin to its cell membrane receptor, the monosialo-ganglioside GM1 (Holmgren et al., 1973; van Heyningen, 1974; Critchley et al., 1981).

Originally, only the A subunit was believed to penetrate the cell membrane in order to activate adenylate cyclase (Bennett & Cuatrecasas, 1977) but more recent work has indicated that the B subunit may also enter the cell (Tsuru et al., 1982). In fact, it has been proposed that the B subunits form a channel through which the A subunit passes in an unfolded form, perhaps with A; leading the way (Antoine et al., 1974). There is in any case a delay between toxin binding and fluid loss (Carpenter et al., 1968).

Adenylate cyclase is activated by the transfer of ADP-ribose from NAD+ to the GTP-binding protein which acts as a regulator (Gill, 1977), possibly at an arginine residue. Nucleoside triphosphate, NAD+, an unidentified cytosolic protein of molecular mass, 15000-20000 daltons and a thiol compound that can reduce the disulphide link in the A subunit are all required for toxin activation of the regulatory protein in pigeon erythrocyte ghosts (Enomoto & Gill, 1979). CT transfers ADP-ribose to the regulatory protein only if it is binding GTP or an analogue (Sternweis et al., 1981; Ward & van Heyningen, 1982). This suggests that the conformation of the protein is important in determining whether ADP-ribosylation occurs. The regulatory protein is thereby prevented from exercising its GTPase activity resulting in permanent activation of the enzyme; thus cyclic AMP levels are raised, absorption of Na+ and Cl− is reduced and secretion of HCO3− increased (Field, 1979).

A number of other GTP-binding proteins will also accept ADP-ribose from CT including tubulin (Hawkins & Browning, 1982) and retinal GTPase (Cooper et al., 1981). CT itself will incorporate ADP-ribose in the absence of any acceptor protein (presumably the prerequisite for a group transfer), and indeed will hydrolyse NAD+ with water as the acceptor, i.e. it has NAD+ glycohydrolase activity (Moss et al., 1979b).

Thyrotrophic hormone, luteinizing hormone and follicle-stimulating hormone, which all have sequence homologies with the CT A subunit (Ledley et al., 1976; Mullin et al., 1976) will also accept ADP-ribose (Trepel et al., 1981).

Adrenocorticotrophic hormone (ACTH) 1–24, which does not share a sequence homology with CT is an excellent acceptor of ADP-ribose. The ADP-ribosylation of proteins may thus require only an accessible arginine as acceptor as indicated by Watkins et al. (1981) in studies with cultured human skin fibroblasts. ADP-ribosylation of membrane proteins accompanies adenylate cyclase activation in other cell types such as brain synaptosomes (Berrillier et al., 1982), fat-cell ghosts (Malbon & Gill, 1979) and pigeon erythrocytes (Enomoto & Gill, 1980).

E. coli enterotoxin

Various strains of E. coli that are responsible for severe diarrhoea in man (Black et al., 1980) and in animals (Porter & Linggood, 1983) elaborate a plasmid-encoded toxin, recently purified and partially characterized (Clements & Finkelstein, 1979; Clements et al., 1980; Gill et al., 1981). The various heat-labile toxins may vary in size, immunological reactivity and amino acid composition but, in general, they show a broad similarity to each other and to CT, notably in subunit composition, ganglioside receptor (Donta & Viner, 1975; Field, 1979; Moss et al., 1981), ability to activate intestinal adenylate cyclase (Gill et al., 1976) and raise intracellular cyclic AMP levels (Kantor et al., 1974). The earlier work was carried out with cell-free supernatants from E. coli, and not with purified toxin, but this is no reason to doubt the relevance of the data. Like CT, LT will catalyse ADP-ribosylation of a rat liver membrane protein (Tait et al., 1980) of M, 11000—rather less, however, than that reported for CT (42000, Doberska et al., 1980; 55000, Beckner & Blecher, 1981).

Recent indications are that LT can bind to glycoprotein receptors in the rabbit small intestine, which do not recognize CT, and activate fluid secretion. CT, however,
still produces a greater level of fluid secretion than LT, so it is not a question merely of the number of available receptors that determines the intensity of the response (Holmgren et al., 1982). Another major difference between the toxins is that LT is activated by disulphide bond reduction and fission of a peptide link to yield the A1 peptide whereas in CT it is only a disulphide link that has to be broken.

**Arginine as the acceptor**

The evidence for arginine as the acceptor amino acid in the regulatory component of adenylate cyclase is rather more circumstantial. A number of basic amino acids were tested for their ability to facilitate the hydrolysis of NAD\(^+\) by CT. Only arginine was found to be effective. At the same time guanidine was shown to be less active and arginine methyl ester more active than the amino acid (Moss & Vaughan, 1977). ADP-ribose-\(\alpha\)-L-arginine was formed (Moss & Vaughan, 1977; Oppenheimer, 1978), not the \(\beta\)-anomer which one might anticipate from a study of calf spleen NAD glycohydrolase (Schuber et al., 1978). Arginine activated LT catalysis in a similar fashion (Moss et al., 1979a). Polyarginine is an excellent acceptor for ADP-ribose (Lai et al., 1981). Our own results with phenylglyoxal, which preferentially reacts with arginine residues, are also consistent with this view (Tait, 1981). It should be noted that water is an acceptor, albeit a weak one, and therefore an activated serine residue might also accept ADP-ribose.

**Studies on other acceptors**

In view of the evidence above we concentrated our search for ADP-ribose acceptors on compounds having guanidine attached to hydrophobic moieties. This stemmed from a consideration of the nature of the toxin's natural substrate (a stalked intrinsic membrane protein, Hudson et al., 1981).

In order to detect acceptor activity we identified the following considerations:

1. An acceptor should increase the rate of nicotinamide release from NAD\(^+\) catalysed by CT in the absence of other known acceptors, i.e. should activate apparent NAD\(^+\) glycohydrolase activity. This property was used as the primary criterion by which potential toxin substrates were identified.

2. An effective ADP-ribose acceptor should inhibit the action of CT on adenylate cyclase systems by competitively diverting the activity of the toxin away from its natural substrate. The inhibitory potency in this system should correlate closely with the ability to function as an NAD\(^+\) glycohydrolase activator.

3. An acceptor should have little or no effect on adenylate cyclase which has been pre-activated by treatment with CT in the absence of the acceptor. Similarly it should have little effect on other forms of adenylate cyclase such as guanine nucleotide-stimulated or fluoride-stimulated enzyme.

**Results:**

Fig. 1. shows that when

\[ R = \begin{array}{c}
\text{OCH}_{2} \text{CH}_{2} \text{NH} \\
\text{OCH}_{2} \text{CH}_{2} \text{NH}
\end{array} \]

functions as an NAD\(^+\) glycohydrolase activator.

Incubations were conducted in a total volume of 100\(\mu\)l containing 200mM-potassium phosphate, pH6.5, 20mM-dithiothreitol, 0.2mM-NAD\(^+\) (containing 50mCi of \([4-\text{\textsuperscript{3}}\text{H}]\text{NAD}\(^+\))\), 100\(\mu\)g CT/ml and 2mM concentrations of the indicated compounds. After 30 min at 37\(^\circ\)C, reactions were terminated by adding 300\(\mu\)l of butan-1-ol and mixing. After centrifugation at 1500\(\times\)g for 5 min, \([4-\text{\textsuperscript{3}}\text{H}]\text{nicotinamide}\) in the supernatant was determined by scintillation counting. Results are expressed as fold-stimulation of the rate of nicotinamide release from NAD\(^+\). The control activity (in the absence of any test compound) was 0.2nmol of nicotinamide released/min per mg of toxin. Values are the means of triplicate determinations.

(i) Compounds in which the length of an unbroken alkyl chain preceding a guanidine group is short (less than four methylene groups), little or no enhancement of cholera toxin activity is observed, regardless of the nature of the R grouping.

(ii) High activity is found with compounds having eight to 12 methylene groups in an unbroken alkyl chain preceding a guanidine group. Benzyl or \(p\)-methoxybenzyl derivatives are usually the most potent.

(iii) Intermediate activity is found in compounds having four to eight methylene groups in an unbroken alkyl chain.

There is therefore a close correlation between enhancement of cholera toxin activity and the length of unbroken alkyl chain preceding a guanidine group in the acceptor molecule. Overall hydrophobicity of the molecule is therefore likely to play a major role in determining recognition as a toxin substrate.

Further studies on a compound with

\[ R = \begin{array}{c}
\text{OCH}_{2} \text{CH}_{2} \text{NH} \\
\end{array} \]
pounds are considerably more effective than either arginine or arginine methyl ester; cholera toxin specific activities in the NAD+-splitting system previously attainable only with 

![Fig. 2. Specificity of compound no. 8 as an inhibitor of the action of CT on adenylate cyclase](image)

All adenylate cyclase assays were conducted in a total volume of 100 µl containing crude rat liver plasma membranes (75 µg protein), 20 mM-4-morpholinepropanesulfonic acid (MOPS), pH 7.5, 5 mM-MgCl₂, 1 mM-cyclic AMP, 1 mM-dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.1 mM-ATP (containing 2 µCi of [α-³²P]ATP), 0.1 mM-isobutylmethylxanthine, 5 mM-creatine phosphate, 10 units/ml adenosine deaminase and either 0.1 mM-GTP or 0.1 mM-Gpp(NH)p. In experiments with CT, membranes were pre-incubated before assay in a medium containing 20 mM-MOPS, pH 7.5, 0.5 mM-GTP, 1 mM-NAD, 5 mM-dithiothreitol, 5 mM-MgCl₂ and 10 µg-CT/ml. After 30 min at 30°C, toxin action was terminated by adding 1 ml of ice-cold 20 mM-MOPS, pH 7.5, 50 mM-sucrose and centrifugation at 1500 g for 10 min. Membrane pellets were resuspended in 80 µl of the above buffer before addition of 20 µl of cyclase assay cocktail. All assays were conducted at 30°C for 10 min and terminated by adding 1 ml of 0.2% sodium dodecyl sulphate followed by 50 µl of [³²P]cyclic AMP as a recovery marker. [³²P]cyclic AMP was isolated by sequential Dowex-Alumina chromatography as described by Salomon (1979) and quantified by liquid scintillation counting. For determination of basal (○), Gpp(NH)p-stimulated (△) and CT-stimulated (■) activities, the test compound was present at the indicated final concentrations in the assay mixtures. For determination of the effect of the test compound on the action of CT (◇), it was present at the same concentrations in the toxin pre-incubation mixtures only.

![Fig. 3. Correlation between the substrate specificities of CT and LT](image)

Nicotinamide release from [4-³²H]NAD in the presence of 2 mM concentrations of the indicated compounds and either 100 µg/ml CT or LT was determined exactly as described in the legend to Fig. 1. LT was pre-activated before use by incubating at 37°C for 30 min in the presence of 40 mM-dithiothreitol and 10 µg/ml trypsin. The action of trypsin was terminated by adding phenylmethylsulphonyl fluoride (50 mM in ethanol) to a final concentration of 0.5 mM. G is --NH→C--NH₂.
Genetics of enterotoxin production in *Escherichia coli*

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Certain strains of *Escherichia coli* have the ability to adhere to and colonize the small intestine of humans and animals and produce enterotoxins. The enterotoxins that will be considered are heat-labile enterotoxin (LT) and the different heat-stable enterotoxins STα (ST1), STβ (ST2), and STγ (ST11). STγ is methanol-soluble and is positive in the infant mouse assay but negative in the weaned pig gut loop test. STα is methanol insoluble and is positive in the pig gut loop test.

The genes coding for the production of these enterotoxins have been located on plasmids, apart from a strain of human origin in which the LT genes are phase-determined (for a review see Elwell & Shipley, 1981). Enterotoxin (or Ent) plasmids have been divided into the following types based on the enterotoxin(s) determined by the plasmid: LT, STα, STβ, STβ-LT, and STγ-LT. STγ plasmids have been found in strains of bovine, porcine, and human origin whereas STγ and STγ-LT plasmids have only been identified in porcine strains (Smith & Halls, 1968; Gyles et al., 1974). LT and STγ-LT plasmids have been detected in *E. coli* from humans (Scotland et al., 1979; McConnell et al., 1980). Many of the Ent plasmids of human origin also code for production of adhesive factors such as colonization factor antigens I and II (for a review see Gastra & de Graaf, 1982). In strains of animal origin the genes for toxin production and adhesion are usually located on separate plasmids, although linkage of the K99 and ST genes on the same plasmid has been observed in some cases. In enterotoxigenic strains resistant to antimicrobial drugs the two properties of toxin production and drug resistance are usually determined by separate plasmids but in a few cases a single plasmid codes for enterotoxin production and drug resistance. One of these plasmids, pCG86, was used for isolation of mutants affecting enterotoxin production.

Abbreviations used: Ent, enterotoxin; LT, heat-labile enterotoxin; ST, heat-stable enterotoxin.

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