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Binding of NAD^+ by cholera toxin

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1. The K_m for NAD^+ of cholera toxin working as an NAD^+ glycohydrolase is 4 mM, and this is increased to about 50 mM in the presence of low-M_r ADP-ribose acceptors. Only molecules having both the adenine and nicotinamide moieties of NAD^+ with minor alterations in the nicotinamide ring can be competitive inhibitors of this reaction. 2. This high K_m for NAD^+ is also reflected in the dissociation constant, K_d, which was determined by a variety of methods. 3. Results from equilibrium dialysis were subject to high error, but showed one binding site and a K_d of about 3 mM. 4. The A1 peptide of the toxin is digested by trypsin, and this digestion is completely prevented by concentrations of NAD^+ above 50 mM. Measurement (by densitometric scanning of polyacrylamide-gel electrophoretograms) of the rate of tryptic digestion at different concentrations of NAD^+ allowed a more accurate determination of $K_d = 4.0 \pm 0.4$ mM. Some analogues of NAD^+ that are competitive inhibitors of the glycohydrolase reaction also prevented digestion.

INTRODUCTION

Cholera toxin (M_r 84000) shares with several other bacterial toxins the ability to catalyse the ADP-ribosylation of a eukaryotic protein. This activity is responsible through its effects on eukaryotic adenylate cyclase for the intense diarrhoea of clinical cholera (Gill & Meren, 1978; Cassel & Pfeuffer, 1978; van Heyningen, 1982). The toxin is made up of five B subunits (M_r 11500 each) that bind to ganglioside G_{M1} on the surface of susceptible cells (van Heyningen, 1983) and a single A subunit composed of two chains, A1 (M_r 22000) and A2 (M_r 5000), linked by a disulphide bond (Gill & Rappaport, 1979; Mekalanos *et al.*, 1979*b*). After reduction, the A1 chain can catalyse several NAD^+-dependent reactions, including ADP-ribosylation of mammalian proteins (in particular N_s, a regulatory component of adenylate cyclase) and of arginine or arginine derivatives, or the hydrolysis of NAD^+ [NAD^+ glycohydrolase activity (Moss & Vaughan, 1978, 1981)].

The substrate *in vivo*, N_s, is notoriously hard to purify, makes up an average of only 1 part in 10^5 of total cellular protein, is extremely labile and hydrophobic, and requires to be in a certain orientation and in an appropriate membrane for activity (Gilman, 1984). The reaction *in vivo* also requires a number of cofactors, both soluble and membrane-bound (Gill, 1975; Moss & Vaughan, 1977; Kahn & Gilman, 1984). Previous studies of the kinetic mechanism of cholera toxin have therefore used model systems based on low-M_r analogues of N_s such as agmatine (Osborne *et al.*, 1985), guanyl-[^{125}I]iodotyramine (Mekalanos *et al.*, 1979*a*) and other substituted guanidine derivatives (Tait & Nassau, 1984; Soman *et al.*, 1986). The kinetic mechanism has been variously reported to follow ordered or sequential mechanisms, although all reports agree on the K_m for NAD^+ being high, in the region of 5 mM (Mekalanos *et al.*, 1979*a*; Osborne *et al.*, 1985).

The present paper is about more accurate determinations of some of the kinetic constants of the toxin and its binding constant for NAD^+. Several experimental problems arose, chiefly because of the high NAD^+ concentrations needed to saturate the binding site.

EXPERIMENTAL

Materials

Cholera toxin was bought either from Sigma Chemical Co., Poole, Dorset, U.K., or from List Biological Laboratories, Campbell, CA, U.S.A. Samples of isolated cholera toxin A1 chain were kindly given by the Institut Mérieux, Charbonnières les Bains, France, and purified by the method of Lai *et al.* (1976). [4-^{3}H]NAD^+ was from Amersham International, and Spectrapor dialysis membranes were from Spectrum Medical Industries, Los Angeles, CA, U.S.A. Compounds A599 and A603 were obtained from Glaxo Group Research, Greenford, Middx., U.K. Other reagents were from Sigma Chemical Co.

Assay of ADP-ribosylation

ADP-ribosylation of low-M_r arginine derivatives was assessed indirectly by measuring the rate of [^{3}H]nicotinamide release from [4-^{3}H]NAD^+ by the method of Tait & Nassau (1984), in which nicotinamide is separated from the reaction mixture (200 mM-potassium phosphate buffer, pH 6.5, containing 20 mM-dithiothreitol, 2 mM-A599 and 100 μg of toxin/ml) by chromatography on QAE- (quaternary aminoethyl-) Sephadex A-25 (Pharmacia, Uppsala, Sweden). Controls were included to take account of nicotinamide formed both by the NAD^+ glycohydrolase activity of toxin preparations and by non-enzymic breakdown of NAD^+.

Equilibrium dialysis

Equilibrium dialysis was carried out in Teflon cells (Dianorm R; Fisons, Loughborough, Leics., U.K.). Each unit had two half-cells (volume 0.25 ml, membrane surface area 2.0 cm^2), separated by a Spectrapor membrane of nominal M_r cut-off 6000–8000. The cells were gently rotated in a water bath at 37 °C. Potassium phosphate buffer (200 mM), pH 6.5, containing 20 nCi of

Abbreviations used: compound A599, {12-[(phenylmethyl)amino]dodecyl}guanidine; compound A603, [10-(acetamido)decyl]guanidine.

[4-^{3}H]NAD$^+$ and unlabelled NAD$^+$ up to 100 mM was injected into one half-cell, and the same volume of buffer containing 10–80 μM cholera toxin into the other.

Digestion of the A1 chain by trypsin

Samples containing 50 μM A1 chain in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.2 M-NaCl, 10 mM-EDTA and various concentrations of NAD$^+$ were left to stand for 10 min at room temperature. Trypsin was added to a final concentration of 5 μg/ml and digestion was allowed to proceed at 37 °C for various times before being stopped by the addition of phenylmethanesulphonyl fluoride in propan-1-ol to a final concentration of 1 mM. Control samples contained ovalbumin instead of A1 chain; their digestion pattern was unaffected by the NAD$^+$. The digested protein was then run on a 15% polyacrylamide gel in the presence of 0.1% (w/v) SDS (Laemmli, 1970) and stained with Coomassie Brilliant Blue R-250. The intensities of the resulting stained bands were measured in a Gilford densitometric scanner.

To determine whether the substrates of cholera toxin have any direct effect on the activity of trypsin, the rate of hydrolysis of 250 μM-*N*-benzyl-α-arginine ethyl ester in 1.0 mM-sodium phosphate buffer, pH 7.0, by trypsin (30 μg/ml) was measured with an automatic titrator in the presence and in the absence of 40 mM-NAD$^+$ or -compound A603. There was no difference in the rates.

RESULTS

Kinetics

NAD$^+$ glycohydrolase. The rate of release of nicotinamide from NAD$^+$ catalysed by cholera toxin in the absence of any acceptor other than water is a measure of the glycohydrolase activity of that preparation of toxin. Fig. 1 shows a plot of such data from which a Michaelis constant, K_m, of 4.0±0.4 mM and a catalytic-centre activity, $k_{cat.}$, of 0.1 s^{-1} were calculated [assuming that all the toxin in solution was active, and using the non-parametric method of Cornish-Bowden & Eisenthal (1978) fitted on the computer by the method of Marquardt (1963)].

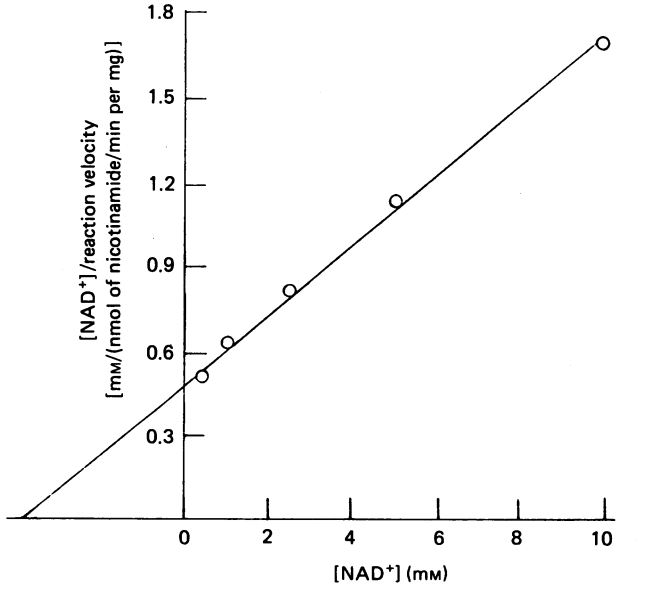

Fig. 1. Kinetic analysis of the NAD$^+$ glycohydrolase reaction catalysed by cholera toxin

The release of [^{3}H]nicotinamide from [^{3}H]NAD$^+$ was measured as described in the text.

ADP-ribosylation reaction. Subsequent experiments were performed with the ADP-ribose acceptor molecules introduced by Tait & Nassau (1984), which are arginine analogues. Most experiments were done with compound A599, but results with compound A603 were in general the same. The K_m for NAD$^+$ was determined by computer analysis of kinetic experiments (as above) to be in the range 45–51 mM, and the K_m for compound A599 to be 0.4 mM. The $k_{cat.}$ in the presence of compound A599 was 4.9 s^{-1}. This indicates that the affinity of the toxin for NAD$^+$ has been decreased by the presence of the artificial acceptors, even though the turnover rate has increased. Numerous attempts were made to obtain enough results for a more detailed analysis of the two-substrate kinetics of the reaction, but this turned out to be impossible for a number of reasons, including increased non-enzymic hydrolysis of NAD$^+$ at high concentration, difficulty with the assay at high ionic strength and the tendency of the artificial substrates to form micelles to an unknown extent so that their effective concentration was unclear.

Competitive inhibitors

Initial rates of ADP-ribosylation were measured in the presence of fixed concentrations of a number of partial structural analogues of NAD$^+$ that have minor alter-

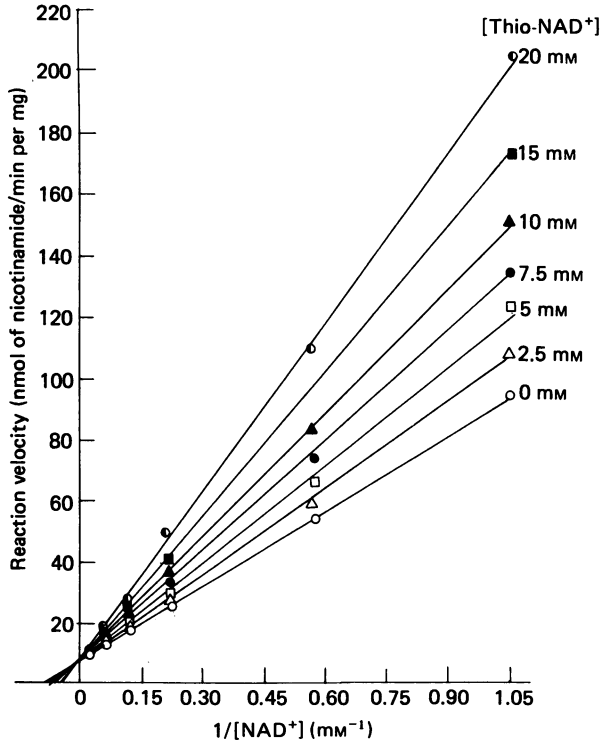

Fig. 2. Double-reciprocal plot of toxin-catalysed ADP-ribosylation in the presence of thio-NAD$^+$

Conditions were similar to those of Fig. 1 with 2 mM-compound A599.

Table 1. Inhibitors of cholera toxin competitive with NAD+

Abbreviations: APAD, acetylpyridine–adenine dinucleotide; deamino-NAD+, nicotinamide–hypoxanthine dinucleotide; deamido-NAD+, nicotinic acid–adenine dinucleotide. The errors given are standard deviations.

Compound	K_i (mM)
Thio-NAD+	16±3
APAD	17±8
Deamino-NAD+	58±18
3-Amino-NAD+	56±18
NMN	> 100
Deamido-NAD+	> 100
ADP-ribose	> 1000
Adenosine	> 1000
Adenine	> 1000
Nicotinamide	> 1000

ations to the nicotinamide ring or adenine ring. Results obtained with one of the inhibitors, the thionicotinamide derivative of NAD+, are presented in Fig. 2 as a double-reciprocal plot. Analysis of these data (as above) showed that the inhibitor constant, K_i, for thio-NAD+ was 16±3 mM. Table 1 shows similar values for several other compounds, all of which were shown to be competitive with NAD+, in that the maximum velocity was unchanged.

Equilibrium dialysis

An initial attempt to measure the binding of NAD+ to the toxin was made by using equilibrium dialysis. Samples of toxin were dialysed to equilibrium (shown to take about 120 min). After this treatment the toxin retained 80–100% of its original activity, and less than 5% of the NAD+ had been hydrolysed, showing that the NAD+ glycohydrolase activity of the toxin need not complicate interpretation of these experiments.

The binding parameters calculated from the equilibrium concentrations of bound and free ligand by the method of Nimmo *et al.* (1977) were (means±S.D.): number of binding sites, $n = 1.0 \pm 0.8$, and dissociation constant, K_d, = 3±2.8 mM. The error involved in these estimates is high, and makes it doubtful whether the values, especially of K_d, can be considered useful. These errors may be due in part to the high ligand concentrations used (which means that the concentration of bound ligand accounts for a very small difference between two large numbers). For example, at the lowest concentration of ligand, 0.1 mM-NAD+, an experimental error in estimating free ligand radioactivity of 1% could lead to errors in the fractional saturation of 4%. At the highest concentration (5 mM-NAD+) the error would be about 20%. So this method gives only a very approximate result.

Similar experiments were performed with A1 chain purified by the method of Lai *et al.* (1976), but these were not successful because the A1 chain can only be kept in solution at useful protein concentrations (> 1 mg/ml) in urea at 2.5 M or higher.

Protective effect of NAD+ on tryptic digestion

An entirely different way of determining K_d came from our observation of a protective effect of NAD+ on proteolytic digestion of the toxin.

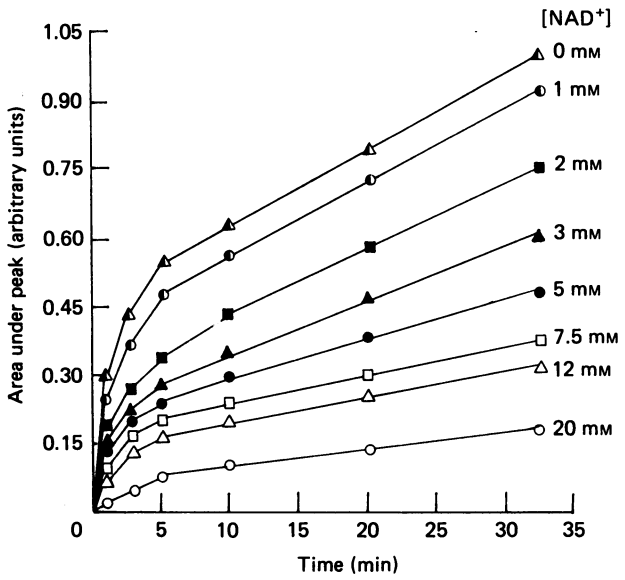

Fig. 3. Rate of digestion of the A1 chain of cholera toxin by trypsin

Digestion at different concentrations of NAD+ was measured by polyacrylamide-gel electrophoresis in the presence of SDS as described in the text.

The A1 chain of cholera toxin gave a clear and consistent pattern of bands when digested with trypsin and analysed by polyacrylamide-gel electrophoresis in the presence of SDS. NAD+ (50 mM) completely protected the A1 chain from this digestion. This was taken as evidence that the NAD+ was inducing a conformational change in the A1 chain upon binding that drastically altered its susceptibility to proteolytic digestion.

A method based on using changes in the rate of tryptic digestion at different ligand concentrations was used successfully by Jacobs & Cunningham (1968) to calculate binding constants for the interaction of creatine kinase with its substrates. Jacobs & Cunningham (1968) used a pH-stat to measure the rate of digestion of creatine kinase. There was not enough cholera toxin available to use this method, so in the experiments described in the present paper the digestion was monitored by using gel electrophoresis.

Analysis of the products of tryptic digestion by gel electrophoresis showed that a clear pattern of bands appeared, ranging in M_r from about 20000 to 12000. The appearance of the largest band, of M_r 20000, was measured in each case. This appearance was more easily measured than the disappearance of the original A1 chain (M_r 22000). However, results from the disappearance of the 22000-M_r band or from an average of the appearance of the bands at M_r 18000, 16000 and 14000 gave essentially the same value for the rate of digestion over 30 min.

To determine the optimum conditions for digestion, samples of the A1 chain were incubated with 1–50 μg of trypsin/ml for times up to 1 h. The clearest pattern of digested bands was obtained with 5 μg of trypsin/ml with digestion continued for up to 30 min. Whole cholera toxin was not used for these experiments, as the pattern of digestion bands became too complicated.

A time course of the digestion of the A1 chain by trypsin at various concentrations of NAD+ is shown in

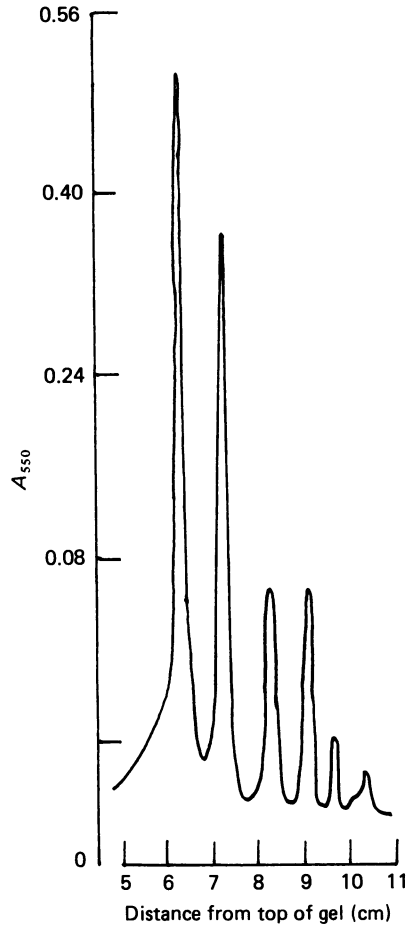

Fig. 4. Densitometric trace of a tryptic digest of cholera toxin

A 25 μg portion of A1 chain was digested with trypsin (5 μg/ml) in the absence of NAD^+ as described in the text.

Fig. 3, and Fig. 4 shows a densitometric scan of a typical lane from a gel electrophoresis of the protein digest. There was a rapid initial burst of digestion, followed by a much slower reaction, which went on at a relatively constant rate for at least 20 min (Fig. 5). It is the slope of the linear portion of this graph that has been used to compare the susceptibility of the protein to tryptic digestion under different conditions. The reason for the initial rapid burst of digestion is not clear, although its size is clearly related to the concentration of NAD^+. It could be caused by a rapid digestion of particularly susceptible bonds, followed by a lower but relatively constant rate of digestion. Alternatively, the linear portion could represent a steady state as the protein of M_r 20000 is formed and degraded. When digestion was allowed to proceed beyond 30 min, the 20000-M_r band gradually disappeared as it was digested to form small peptides. Whatever the complex kinetic explanation behind the biphasic digestion rate [which was also observed by Jacobs & Cunningham (1968)], the linear portion is clearly a function of, and related to, the NAD^+ concentration.

NAD^+ (50 mM) inhibited digestion of the A1 chain completely for 1 h at 37 °C. NAD^+ also protected the A1 chain from digestion by chymotrypsin (5 μg/ml) but to a lesser extent, with some digested material apparent after a few minutes, even in the presence of 50 mM-NAD^+.

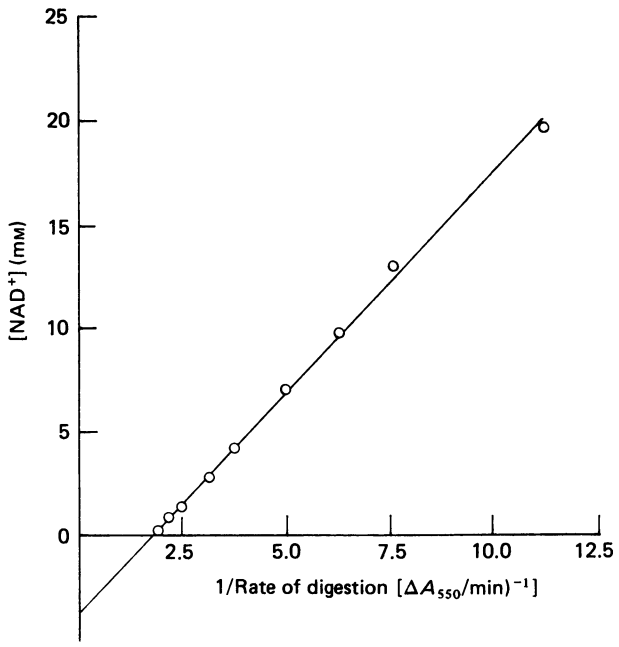

Fig. 5. Secondary plot of NAD^+ concentration and the rate of digestion of the A1 chain

Digestion was determined from the plots shown in Fig. 3, and analysed as described in the text.

When the A1 chain was digested with trypsin in the presence of 10 mM-compound A603, there was essentially no digestion over 30 min. Digestion was inhibited by concentrations of compound A603 down to 0.5 mM (the lowest concentration tested). This shows that compound A603 can bind to the toxin A1 chain in the absence of NAD^+. Analysis of the data (not shown) allowed only a rough estimate of the dissociation constant, of about millimolar.

Quantitative relationship between digestion rate and NAD^+ binding

A binding constant can be calculated from the measured digestion rates at different concentrations of NAD^+ by the following treatment.

Consider a ligand, L, binding to protein, P, where:

$$L + P = PL$$

The dissociation constant for the ligand, K, is given by:

$$K = [P][L]/[PL]$$

$$[P] = K[PL]/[L]$$

Assume that P only is digested and PL not at all (i.e. in this case that NAD^+ protects from digestion completely), and that the velocity of digestion, v, is given by:

$$v = k[P]$$

$$V_{max.} = k[P_{max.}] = k[P_t]$$

where $[P_t]$ is the total concentration of all forms of protein. This $V_{max.}$ is the velocity observed when $[L] = 0$.

$$[PL] = [P_t] - [P]$$

$$v = k[P] = kK[PL]/[L] = kK([P_t] - [P])/[L]$$

$$v = k[P_t] \cdot K/[L] - k[P] \cdot K/[L]$$

$$v = V_{max.} \cdot K/[L] - vK/[L]$$

Multiplying by [L] and dividing by v gives:

$$[L] = V_{max.} \cdot K/v - K$$

A graph of [L] versus $1/v$ is a straight line with intercepts $-K$ and $1/V_{max.}$ and slope $V_{max.} \cdot K$. If $[L_{total}] \gg [P_t]$, then [L] can be replaced by $[L_{total}]$, which is actually plotted.

Fig. 5 shows such a plot of NAD^+ concentration and the rate of digestion of A1 chain. The calculated value of K_d is 4.0 ± 0.4 mM.

Binding of NAD^+ analogues

The ability of various analogues of NAD^+ to protect the A1 chain from digestion by trypsin in place of NAD^+ was studied. Samples of A1 chain (25 μg) in the same buffer as before at pH 7.5 were left to stand for 10 min at room temperature with either 10 mM or 20 mM additions of the following: thio-NAD^+, deamino-NAD^+, the acetylpyridine analogue of NAD^+, nicotinamide, NMN, $NADP^+$, NADH, ADP-ribose, ATP, ADP, AMP and adenine. The only molecules capable of protecting the A1 chain from digestion by trypsin to any extent were thio-NAD^+ and the acetylpyridine analogue of NAD^+ at 10 mM and above, and deamino-NAD^+, nicotinamide and NMN at 20 mM. NADH could also protect at 10 mM, but this may be due to contamination with, or partial oxidation to, NAD^+; no protection was observed with a fresh batch of NADH.

DISCUSSION

Enzyme activity

Our K_m value of 4 mM for the NAD^+ glycohydrolase activity of the toxin agrees well with published results (Mekalanos *et al.*, 1979*a*; Moss *et al.*, 1979). It is increased to around 50 mM in the presence of artificial ADP-ribose acceptors, again a result in agreement with that obtained by Osborne *et al.* (1985), who used agmatine, a different acceptor, and who showed that binding of either substrate lowered the affinity for the other, leading them to suggest a random-order sequential binding mechanism for the enzyme toxin. It is surprising that binding these acceptors should decrease the affinity of the toxin for NAD^+ so much. Possibly, although they can bind, they cannot induce the conformational change that the natural substrate, the N_s protein of adenylate cyclase, can.

Binding experiments

Equilibrium dialysis is the most obvious method to use for binding experiments. It could not be used in a study of binding of NAD^+ by diphtheria toxin because of the high NAD^+ glycohydrolase activity of that toxin (Kandel *et al.*, 1974). However, the glycohydrolase activity of cholera toxin is very much lower [and may in some cases be due to contaminants rather than intrinsic to the toxin (Tait & van Heyningen, 1978)], so this was not a problem in this case. However, it is impossible to work at toxin concentrations greater than about 1 mg/ml because of precipitation problems, and, at the millimolar concentrations of NAD^+ required, this meant that the experimental results had too great error to give more than order-of-magnitude estimates of K_d. Radiolabelled compound A603 or A599 is not available, so equilibrium-dialysis experiments with these substrate analogues were not attempted.

Digestion with trypsin

Substrate protection from proteolysis has been reported for other ADP-ribosylating toxins (Kandel *et al.*, 1974; Tweten *et al.*, 1985) and for other unrelated proteins (e.g. Jacobs & Cunningham, 1968; Trayser & Colowick, 1961), and has been exploited successfully in the calculation of binding constants.

The protection elicited by the two substrates in our experiments was not additive, implying that there are some similarities in the conformational change that they each bring about, perhaps in the same part of the molecule. The K_d value of about 4 mM calculated from the secondary plot of digestion rates (Fig. 5) agrees well with the K_m for NAD^+. The ability of various NAD^+ analogues and derivatives to protect the A1 chain from digestion (which must be a tentative measure of binding affinity) also correlates with their K_i values, in that, for example, the whole of the NAD^+ molecule is needed.

The ability of compound A603 to bind to the A1 chain in the absence of NAD^+ and vice versa, especially when taken together with kinetic evidence, points to a random-order sequential mechanism for the toxin. This mechanism has also been proposed for an avian erythrocyte transferase (Osborne *et al.*, 1985), but diphtheria toxin and *Pseudomonas* exotoxin A both seem to have compulsory-order mechanisms (see van Heyningen, 1980).

Nature of the binding site

What, if anything, do these results say about the nature of the binding sites for NAD^+ and for compound A603? The low affinity for NAD^+ is closer to that for dehydrogenases (typically 10^{-4}–10^{-3} M; Dalziel, 1975) than for other ADP-ribosyltransferases (usually around 10^{-6} M; Osborne *et al.*, 1985; Burtscher *et al.*, 1986) in spite of their very different actions. There is no obvious explanation for this low affinity, which is especially surprising as the intracellular concentration of NAD^+ is around 70–100 μM (Gill, 1975). It is possible that the K_d is quite different in the cell because of the hydrophobic environment of adenylate cyclase, or the effect of other components involved in the action of the toxin, e.g. cytosolic factors (Enomoto & Gill, 1979; Kahn & Gilman, 1984, 1986). There is also the possibility that there is some intracellular compartment where the NAD^+ concentration is particularly high, or, conceivably, that the true substrate is not NAD^+ at all, but some other NAD^+ analogue previously unsuspected. But there is no direct evidence for any of these ideas.

The binding of NAD^+ involves recognition of the whole molecule, with nicotinamide specially important. Similar results have been reported for other toxins and for dehydrogenases. Secondary structures predicted from primary sequences have suggested that the typical NAD^+-binding region of dehydrogenases [an alternating α-helix and β-sheet structure (Rossman *et al.*, 1974)] is found in the cholera and diphtheria families of toxins (Duffy & Lai, 1986; Yamamoto *et al.*, 1984). However, the evidence from X-ray crystallography shows quite clearly that this structure is not found in the ADP-ribosylating *Pseudomonas* exotoxin A (Allured *et al.*, 1986), which is very similar mechanistically to diphtheria toxin. There seems little real evidence for similarities between the dehydrogenases and the ADP-ribosyltrans-

ferases; they both use NAD^+ as a substrate, but in quite different ways.

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