Photolabelling of cholera toxin by NAD⁺

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When cholera toxin is incubated under u.v. light with NAD⁺ labelled in either the adenine or the nicotinamide moiety, radioactivity becomes covalently bound to the protein. The reaction is specific for cholera toxin, and is inhibited by excess unlabelled NAD⁺ or NAD analogues. Only the active A1 chain of the toxin is labelled. The u.v.-absorption spectrum of the product is very similar to that of NAD⁺, and shows the same reaction with cyanide. The nature of the product is therefore different from that found when diphtheria toxin is photolabelled [Carroll & Collier (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3307–3311] in that the yield is lower, but both moieties of the NAD molecule become bound.

INTRODUCTION

Cholera toxin is one of a family of toxins (including those of diphtheria and pertussis) that are ADP-ribosyltransferases (for general review see van Heyningen, 1982; Lai, 1986). They catalyse the transfer of ADP-ribose from NAD⁺ to an acceptor protein. The substrate of cholera toxin is the N_s protein of adenylate cyclase. This transferase activity is also found in several enzymes present in normal cells, and increasingly seems likely to be involved in a physiological control mechanism that is taken over by opportunistic toxins.

Cholera toxin has five B subunits and one A subunit [itself two polypeptide chains, A1 (*M_r* 22000) and A2 (*M_r* 5000), joined by a disulphide bond]. The function of the B subunits is to bind to ganglioside molecules in the outer cell membrane, introducing the A1 chain into the cell. It is the A1 chain that has the transferase activity. The present paper is about the identification of the NAD-binding site on the A1 chain. It was first shown by Carroll *et al.* (1980) that diphtheria toxin could be photolabelled to high yield by incubation with NAD⁺ under u.v. light. The nicotinamide portion only of the NAD was covalently bound to glutamic acid residue 148 on the protein, forming an α -amino- γ -(6-nicotinamidyl)butyric acid residue (Carroll & Collier, 1984; Carroll *et al.*, 1985).

In the present paper we show that cholera toxin can also be photolabelled with NAD⁺, although the nature of the reaction is clearly different.

MATERIALS AND METHODS

Materials

Cholera toxin was bought from Sigma Chemical Co., Poole, Dorset, U.K., and radioactive NAD⁺ from Amersham International, Amersham, Bucks., U.K.

Assay of ADP-ribosyltransferase activity

ADP-ribosyltransferase activity was measured by the method of Tait & Nassau (1984), in which nicotinamide is separated from the reaction mixture by chromatography on QAE- (quaternary aminoethyl-)Sephadex A-25.

graphy on QAE- (quaternary aminoethyl-)Sephadex A-25.

Photoaffinity labelling

Solutions of 60 μ M cholera toxin in 50 mM-Tris/HCl buffer, pH 7.5, containing 100 mM-NaCl, 3 mM-NaN₃, 1 mM-EDTA and 20 mM-dithiothreitol were made up to 5–20 mM-NAD⁺ containing 25 μ Ci of [*nicotinamide-4-³H*]NAD⁺ or [*adenine-2,8-³H*]NAD⁺ and were left to stand for 10 min at 4 °C. Samples were then placed as 100–150 μ l droplets on a Parafilm-coated micro-titre plate floating in an ice bath, and irradiated at a distance of 5 cm with a CAMAG 8 W dual-wavelength lamp set at 254 nm. At appropriate times 20 μ l samples were removed and diluted into 6 M-urea containing 25 mg of bovine serum albumin/ml (in order to remove any non-covalently bound NAD⁺). Protein was then precipitated by the addition of 300 μ l of 12% (w/v) trichloroacetic acid in 0.1 M-H₃PO₄, and the pellet was centrifuged down, washed three times with the trichloroacetic acid solution and finally dissolved in 600 μ l of water. Then 10 ml of emulsifying scintillant was added and the radioactivity was measured on a scintillation counter. Larger samples of labelled protein were irradiated in the same way, and loaded directly on to chromatography columns.

Spectra

Spectra were measured in a Cary model 219 spectrophotometer, in which the reference cuvette contained everything except nucleotide.

RESULTS

We have recently shown (T. S. Galloway & S. van Heyningen, unpublished work) that the binding of NAD⁺ to cholera toxin is very weak: the dissociation constant *K_d* is about 4 mM. Diphtheria toxin binds NAD⁺ much more strongly (*K_d* about 4 μ M). This means that the labelling conditions used by Collier and his co-workers (Carroll *et al.*, 1980; Carroll & Collier, 1984) were not useful in this case. Concentrations of NAD⁺ of the order of the *K_d* are needed in order to have a

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substantial occupancy of the binding site. Fig. 1 shows the effect of incubating toxin with 5 mM-NAD⁺ labelled with ³H in the adenine or the nicotinamide moiety for periods of up to 4 h (the Figure shows the result of two separate experiments). Radioactivity from either moiety is incorporated into the protein. Varying the pH from 6.5 to 8.5 had no significant effect on the extent of incorporation. When the NAD⁺ concentration was increased up to 16 mM (Fig. 2), there was a small increase in both the rate and the extent of incorporation. A greater increase may have been prevented by a concomitant increase in optical shielding caused by the absorbance of the NAD⁺ itself at 260 nm. 'Scavengers' in the solution, e.g. Tris, and dithiothreitol should have

Fig. 1. Photoinduced incorporation of NAD⁺ by cholera toxin as a function of time

Samples were treated as described in the text. ○, [nicotinamide-4-³H]NAD⁺; ●, [adenine-2,8-³H]NAD⁺.

Fig. 2. Effect of NAD⁺ concentration of photoinduced labelling of cholera toxin

The conditions were the same as for Fig. 1. ○, 4 mM-NAD⁺; □, 8 mM-NAD⁺; △, 16 mM-NAD⁺.

destroyed all photogenerated intermediates at places other than the binding site, e.g. free in solution. The maximum extent of incorporation was about 0.2 mol of NAD⁺/mol of protein.

Several lines of evidence show that this incorporation was specific, if slow. In general, non-specific binding is not saturable (Browne *et al.*, 1971; Katzenellenbogen *et al.*, 1974; Cooperman *et al.*, 1975), whereas this reaction did show saturation with time. In the presence of 100 mM unlabelled NAD⁺, or of 100 mM-nicotinamide or -acetylpyridine-adenine dinucleotide (both competitive inhibitors of NAD⁺ binding), the extent of incorporation of radioactivity was decreased to background, indicating that the covalent incorporation was occurring at the same site as that binding unlabelled NAD⁺.

The incorporation of NAD⁺ was also specific for cholera toxin. Irradiation of several other proteins (e.g. bovine serum albumin, trypsin), including NAD⁺-binding proteins (e.g. lactate dehydrogenase), under the same conditions showed no incorporation of radioactivity.

Both the toxin and the NAD⁺ remained intact during the experiments. Irradiated toxin (in the absence of NAD⁺) retained 80–100% of the enzymic activity of untreated controls, and irradiated NAD⁺ showed breakdown to free nicotinamide of less than 10%. When NAD⁺ was incubated with toxin for several hours without irradiation, about 5% of it was hydrolysed. The amount of experimentally observed apparently incorporated radioactivity was decreased to the control radioactivity found in the absence of toxin.

It was not possible to determine the activity of the photolabelled toxin because the assays available (see Tait & Nassau, 1984) are neither precise nor sensitive enough to detect a loss of activity of only 10–20% of the sample. Attempts to separate labelled and unlabelled toxin by affinity chromatography on NAD⁺-agarose were unsuccessful.

Location of the photoincorporated NAD⁺ within the toxin

Since it is the A1 chain of cholera toxin that is known to catalyse the ADP-ribosylation, it is important to show that it also contains the NAD⁺-binding site. Samples of toxin photolabelled in either moiety were subjected to polyacrylamide-gel electrophoresis in the presence of SDS, and the resulting gels were autoradiographed. All the radioactivity had migrated with the A1 chain at an apparent *M_r* of 22000. This labelled A1 chain was further purified by chromatography of the reduced toxin photolabelled in the nicotinamide moiety on Sephadex G-75 in 5% (v/v) formic acid (conditions known to separate the toxin into its subunits; Lai *et al.*, 1976). 3 shows that essentially all the radioactivity was associated with a peak shown by electrophoresis and ADP-ribosyltransferase activity measurements to be the A1 chain. These experiments also provide evidence that the binding of NAD⁺ to the protein was covalent, since it withstood exposure to these chaotropic reagents.

Nature of the incorporated NAD⁺

The experiments shown in Fig. 1 show that radioactivity from either the nicotinamide or the adenine moiety became incorporated into cholera toxin, so that all or most of the NAD⁺ molecule became covalently bound to the protein. This is confirmed by spectroscopic

Fig. 3. Isolation of the radiolabelled A1 chain of cholera toxin

A 5 mg portion of photolabelled toxin was applied to a column (1 cm $\times$ 110 cm) of Sephadex G-75 (fine grade) (Pharmacia) in 5% formic acid and developed at 20 ml/h. Fraction volume was 2.0 ml. ●, Radioactivity; ○, concentration of protein.

evidence. A sample of toxin photolabelled in the nicotinamide moiety was digested with trypsin and chromatographed on a column of Sephadex G-25 in 5% formic acid, as shown in Fig. 4. There were two radioactive peaks, both of which yielded a complex mixture of amino acids and derivatives after hydrolysis.

The u.v.-absorption spectrum of the major peak, presumably a peptide containing covalently bound NAD, is shown in Fig. 5: there is a maximum at 260 nm characteristic of the adenine moiety of NAD⁺. When this sample was treated with KCN, a new absorbance peak at about 330 nm appeared, similar to that found on treatment of NAD⁺ with cyanide and analogous to the spectral change when NAD⁺ is reduced to NADH that is caused by substitution into the nicotinamide ring (Colowick *et al.*, 1951). These spectra more closely resemble those of NAD⁺ and its cyanide adduct than those of many other similar compounds (Siegel *et al.*, 1959). NMN, for example, after reaction with cyanide has a similar peak at 325 nm but no peak at 260 nm.

The spectrum of the minor peak was different: it showed a maximum at 264 nm with a shoulder at 256 nm and was unchanged by reaction with KCN. This suggests that the nicotinamide ring was no longer available for reaction. Two rather different photoproducts have been obtained.

The major tryptic peptide isolated by chromatography as described above was further purified by chromatography on Dowex AG50W, but, unfortunately, attempts to characterize it in more detail were not successful: hydrolysis produced a complex mixture of amino acids and NAD⁺ derivatives that could not be further analysed.

Fig. 4. Chromatography of a tryptic digest of the photolabelled A1 chain of cholera toxin

Protein digested as described in the text was applied to a column (2.2 cm $\times$ 90 cm) of Sephadex G-25 (fine grade) (Pharmacia) in 5% formic acid, and developed at 10 ml/h. Fraction volume was 2.0 ml. ●, Radioactivity; ○, concentration of protein.

Fig. 5. Spectrum of a major radioactive peak from the tryptic digest of photolabelled A1 chain of cholera toxin

—, In the absence of cyanide; ----, in the presence of cyanide.

DISCUSSION

These experiments show that it is possible to label a single site in the enzymically active peptide of cholera toxin with NAD⁺. The yield was lower than that found

with diphtheria toxin (Carroll *et al.*, 1980; Carroll & Collier, 1984), but is typical of photochemical labelling reactions in general. In any case the nature of the product is quite different from that found with diphtheria toxin. The relatively low power of the lamp (8 W) may be a reason why such long periods of photoreaction were needed, but there was no sign of any cross-linking of the protein, or of any non-specific labelling.

The binding must be covalent, since the protein-NAD⁺ bond remained stable in 12% (w/v) trichloroacetic acid, 5% formic acid, 6 M-urea, 0.1% SDS and other chaotropic reagents, and was also unaffected by trypsin.

Two slightly different products seem to have been formed: one containing most of the NAD⁺, and one (present in much smaller amounts, and found in only one of several similar experiments) in which the nicotinamide ring was unavailable for reaction, at least with cyanide, although its radioactivity and spectrum showed that some of the ring must still have been present. It could be that this material is some breakdown product of the first, or perhaps there was a different photoreaction in the first place.

In the light of the specificity of labelling, the great probability must be that this NAD⁺-binding site is the true binding site of the toxin, about which rather little is known. But it remains possible that the residue affected is an artifact of the photolabelling. It is remarkable that two different ADP-ribosyltransferases can be labelled in this way, when other NAD⁺-binding enzymes cannot.

The nature of the binding is presumably connected in some way to the reaction catalysed.

We are grateful to the Medical Research Council for a grant (to S. V. H.) T. S. G. was an S.E.R.C. C.A.S.E. student with Glaxo Group Research Ltd.

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