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Thiol reagents are substrates for the ADP-ribosyltransferase activity of pertussis toxin

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Thiols such as cysteine and dithiothreitol are substrates for the ADP-ribosyltransferase activity of pertussis toxin. When cysteine was incubated with NAD^+ and toxin at pH 7.5, a product containing ADP-ribose and cysteine (presumably ADP-ribosylcysteine) was isolated by high-performance liquid chromatography, and characterized by its composition and release of AMP with phosphodiesterase. Cysteine has a K_m of 105 mM at saturating NAD^+ concentration. The ability of thiols to act as a substrate is one explanation for the very high concentrations (250 mM or greater) that have been observed to enhance the apparent NAD glycohydrolase activity of the toxin.

Pertussis toxin; ADP-ribosylation; Thiol; NAD

1. INTRODUCTION

Pertussis toxin (review [1]) is a pathologically important protein secreted by *Bordetella pertussis*, the bacterium that causes whooping cough. The toxin is composed of five different subunits named S-1 to S-5 on the basis of decreasing molecular mass. The S-1 subunit, or A protomer, is an ADP-ribosyltransferase and also has NAD glycohydrolase activity. The remaining subunits constitute the B protomer, and are responsible for binding to a cell surface receptor and facilitating the entry of S-1 into the cell. Once within the cell, S-1 catalyses the transfer of ADP-ribose from NAD^+ to G_i, a GTP-binding regulatory protein of the adenylate cyclase complex. The toxin gene has been sequenced [2,3].

A Michaelis constant (K_m) of 25 μM has been measured for NAD^+ in the NAD glycohydrolase reaction catalysed by the toxin, and generally assumed to be a model for the ADP-ribosyltransferase [4]. It purports to represent the breakdown of NAD^+ with water as the only acceptor for ADP-ribose. It has been found that this reaction is accelerated by sulphydryl compounds such as dithiothreitol, presumably because it is required for reducing a disulphide bond in the S-1 subunit, making it enzymically active [5]. However, the concentrations of thiol used have been much higher than is normally needed for activation (e.g. cholera toxin is analogous in many ways but needs only 10 mM at most). Concentrations of 250 mM dithiothreitol have often been used [4,6].

Here, we show that thiol reagents are substrates for the ADP-ribosyltransferase reaction of the toxin, and that is why high concentrations accelerate the apparent glycohydrolase activity.

2. MATERIALS AND METHODS

2.1. *Materials*

Pertussis toxin was from the Public Health Laboratory Service Centre for Applied Microbiology and Research (Porton, Wiltshire, England) and was purified by the method of Irons and MacLennan [7]. [*nicotinamide*-4-^{3}H]NAD^+ was from

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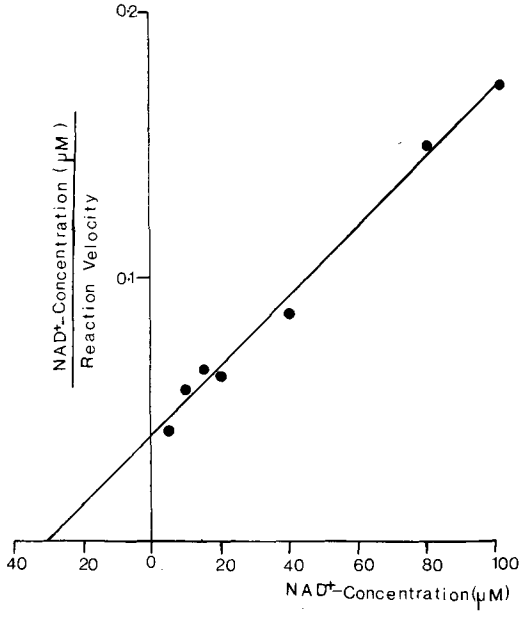

Fig.1. Effect of NAD^+ concentration on the ADP-ribosyltransferase activity of pertussis toxin at 250 mM dithiothreitol. Reaction velocity is measured as pmol nicotinamide released/h per sample.

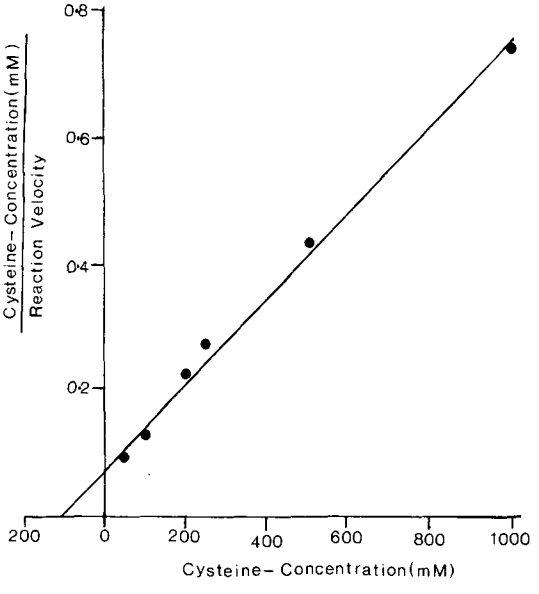

Fig.2. Effect of cysteine concentration on the ADP-ribosyltransferase activity of pertussis toxin at 100 μM NAD^+. Reaction velocity as in fig.1.

Amersham International, and [*adenine*-2,8-^{3}H]NAD^+ and [U-^{14}C]cysteine from DuPont. Snake venom phosphodiesterase was from Sigma.

2.2. *Assay of apparent NAD^+ hydrolysis*

NAD^+ glycohydrolase activity was measured by the method of Moss and Vaughan [8], in which [^{3}H]nicotinamide released from [*nicotinamide*-4-^{3}H]NAD^+ is measured by ion-exchange chromatography on Dowex AG-1X resin. The reaction mixture typically contained 50 mM phosphate buffer, 250 mM dithiothreitol, 1 mg/ml ovalbumin and 12 μg toxin (pH 7.5) in a final volume of 300 μl.

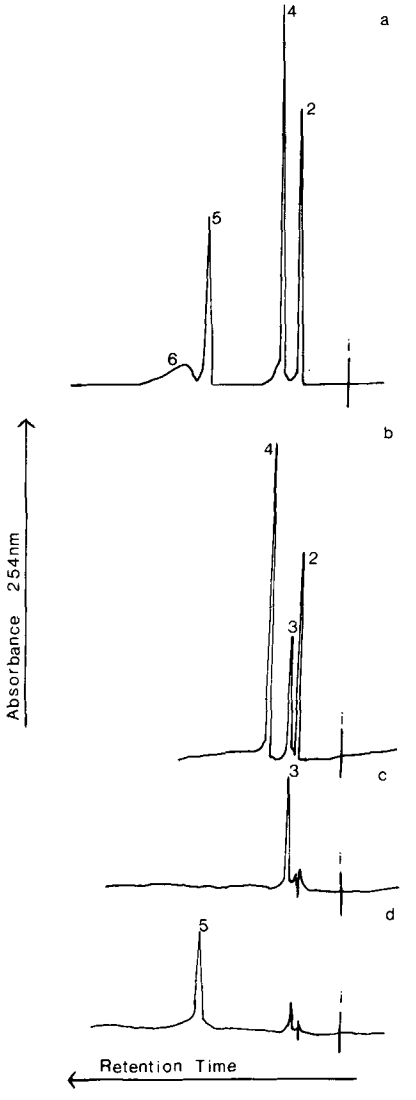

Fig.3. HPLC traces. 1, injection point; 2, ADP-ribose; 3, putative ADP-ribosylcysteine; 4, NAD^+; 5, AMP; 6, nicotinamide.

2.3. *Separation of reaction products by high-performance liquid chromatography*

Reaction products were filtered through an Amicon OM-10 filter to remove protein, and applied to a reverse-phase ODS-HYP 2734 column (25 cm × 4.6 mm) in 10 mM $NH_4H_2PO_4$, 10% MeOH (pH 3.5) run at 1 ml/min, using Altex equipment. The eluate was monitored by its absorbance at 254 nm. 5 drop fractions (about 100 μl) were collected and, where appropriate, assayed for ^{14}C and ^{3}H by scintillation counting.

3. RESULTS

3.1. *Effect of thiol on the rate of NAD^+ hydrolysis*

The rate of release of nicotinamide catalysed by pertussis toxin in the presence of 250 mM dithiothreitol was assayed as in [8]. Typical results are shown in fig.1, and give a Michaelis constant, K_m, of 30 μM for NAD^+.

The rate of nicotinamide release was also assayed at different concentrations of several other thiols, e.g. mercaptoethanol, glutathione and cysteine. In all cases, increasing concentrations of thiol increased the rate of reaction until saturation was achieved at concentrations of the order of 300 mM, suggesting that the thiols were substrates. Fig.2 shows a conventional kinetic analysis of the effect of cysteine concentration at fixed high NAD^+ concentration (100 μM, near saturating). This suggests that cysteine is acting as a substrate of the reaction, showing a K_m of 105 mM.

3.2. *Isolation of an ADP-ribose-sulphydryl compound*

Fig.3a shows the separation by high-performance liquid chromatography of ADP-ribose, NAD^+, AMP and nicotinamide. The products of an incubation of 50 μM NAD^+ with pertussis toxin in the presence of 250 mM cysteine for 4 h at 30°C were applied to such a column, and ADP-ribose and NAD^+ identified in the eluate (fig.3b). (Nicotinamide is much retarded in this system and thus hard to detect at low concentration; cysteine cannot be detected at 254 nm.) Radiolabelled compounds [*adenine*-2,8-^{3}H]NAD^+ (2.5 μCi/μmol in the mixture) and [U-^{14}C]cysteine (1.3 μCi/mmol) were incorporated into the reaction mixture, the experiment repeated, and the solution applied to the HPLC column. Fig.4 shows

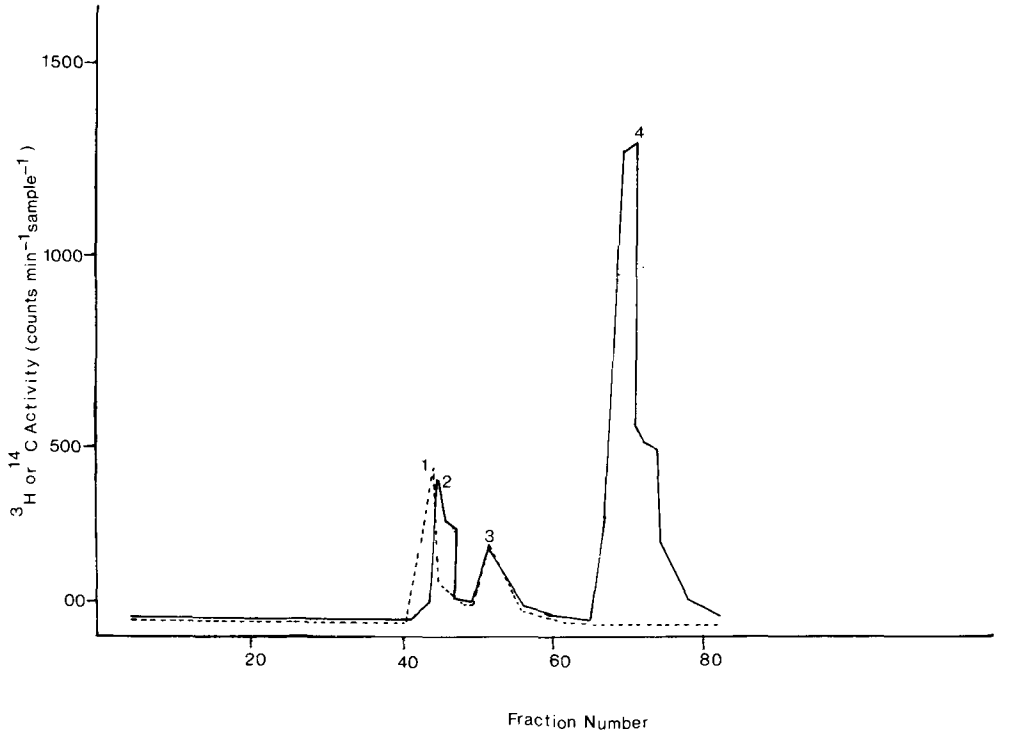

Fig.4. Elution of radioactive material from the HPLC column. 1, cysteine; other peaks as in fig.3. (——) ^{3}H; (– – –) ^{14}C.

the result. It is clear that the unknown product contains both ^{14}C and ^{3}H, and so is a compound made from both cysteine and the adenine moiety of NAD^+. This peak was collected and freeze-dried. Fig.3c shows an HPLC run of a sample.

3.3. *Treatment with phosphodiesterase*

If the unknown compound is ADP-ribosylcysteine, then treatment with snake-venom phosphodiesterase should release AMP, and phosphoribosylcysteine as reaction products. A sample of the purified material (as shown in fig.3c) was incubated with phosphodiesterase from *Crotalus durissus terrificus* (20 μg/ml) in 50 mM phosphate buffer (pH 7.5) for 5 h at 37°C, and applied to a column as shown in fig.3d. Several peaks were identified: one corresponding to the starting material and labelled with both ^{3}H and ^{14}C, one labelled with ^{3}H and corresponding in position to AMP, and another not previously seen but labelled with ^{14}C and in a position compatible with its being phosphoribosylcysteine.

4. DISCUSSION

These experiments show that thiols such as cysteine are substrates for pertussis toxin; that is the reason why such high concentrations have been needed to achieve the maximum rate of breakdown of NAD^+. The K_m value obtained for the apparent NAD glycohydrolase reaction of 30 μM is in reasonable agreement with published values [4], that were determined at high concentrations of dithiothreitol. The K_m for cysteine at saturating concentration of NAD^+ was 105 mM, a high value but one that does show that cysteine can be a useful substrate at the high concentrations that have been used [4,6].

By using NAD^+ and cysteine labelled with different radionuclides, we have been able to show that pertussis toxin can catalyse the formation of a compound containing both the adenine moiety of NAD and the carbon atoms of cysteine. Although this does not prove a particular structure for the product, it is reasonable to suppose that it is ADP-ribosylcysteine with a covalent bond between the C-1 atom of ribose and the sulphur atom of cysteine. Its elution position from the HPLC column is compatible with this structure, and treatment with phosphodiesterase liberated AMP as would be predicted.

Cysteine was identified as the substrate for ADP-ribosylation by toxin on the GTP-binding protein transducin (a model for G_i) by West et al. [9], who also suggested that a glycosyl-cysteine bond had been formed. The observation that cysteine can also be an acceptor for ADP-ribose in vitro makes their observation (which was well supported experimentally) less surprising than it appeared at first sight.

These experiments show that thiols can have two roles in the activity of pertussis toxin in vitro: as an activator of the toxin by reduction of a disulphide bond, and as a substrate for the toxin-catalysed reaction.

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