The purification of a cysteine-dependent NAD⁺ glycohydrolase activity from bovine erythrocytes and evidence that it exhibits a novel ADP-ribosyltransferase activity

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An NAD⁺:cysteine ADP-ribosyltransferase activity was purified from bovine erythrocytes on the assumption that, like pertussis toxin, the enzyme would exhibit a cysteine-dependent NAD⁺ glycohydrolase activity. A three-step purification procedure was developed involving (1) precipitation with 40 % (NH₄)₂SO₄, (2) binding to a cysteine-Sepharose affinity column, and (3) binding to an NAD⁺ affinity column. PAGE showed a single band of *M_r* 45000. The enzyme had been purified 47000-fold and had a specific activity of 1900 nmol nicotinamide released/min per mg. A study of the kinetic properties of this enzyme showed saturation kinetics for cysteine (*K_m* = 4.0 mM). The ability of this enzyme to ADP-ribosylate protein was investigated using re-sealed inverted bovine erythrocyte ghosts. Incubation of the purified enzyme with erythrocyte ghosts and [adenylate-³²P]NAD⁺ led to

the enhanced dose-dependent labelling of several proteins, a doublet of high *M_r* and proteins of *M_r* 60000, 55000 and 29000, identified by autoradiography of separated proteins on SDS/PAGE. The enzyme-catalysed labelling of the major component at *M_r* 55000 was blocked by pre-treatment of the erythrocyte ghosts with *N*-ethylmaleimide, a sulphhydryl alkylating agent, and the label was released by mercuric ion, but not by hydroxylamine. These experiments suggested that a cysteine residue on the target protein had been mono-ADP-ribosylated. This supposition was further supported by identification of the mercuric-ion-released radiolabelled product as ADP-ribose by HPLC, and the observation that free ADP-ribose was unable to modify the membrane target protein directly.

INTRODUCTION

Mono-ADP-ribosyltransferases (E.C. 2.4.2.30 and E.C. 2.4.2.31) catalyse the transfer of a single ADP-ribose unit from NAD⁺ to an acceptor site on the target protein with the concomitant release of nicotinamide and a proton. The most widely studied mono-ADP-ribosyltransferase reactions are those catalysed by bacterial exotoxins, such as those from *Vibrio cholerae*, *Bordetella pertussis* and *Corynebacterium diphtheriae* [1]. Each bacterial exotoxin modifies a specific amino acid of a specific protein target; cholera toxin modifies Arg-201 of G_sα [2] and pertussis toxin modifies Cys-352 of G_iα [3]. However, the reaction is not unique to bacterial exotoxins; there is evidence for endogenous mono-ADP-ribosylation of a variety of cellular target proteins in many different tissues [4]. Endogenous ADP-ribosyltransferase activity has been linked with a variety of cellular processes, e.g. receptor-mediated signal transduction [5,6], calcium signalling [7,8] and growth and differentiation [9–11]. The level of understanding of the nature of these endogenous ADP-ribosyltransferases is poor relative to the extent of knowledge concerning the bacterial toxins. Not all of the target proteins have been identified, and it is not clear whether all of the modifications reported require an ADP-ribosyltransferase to catalyse the reaction, or if they may proceed non-enzymically [12–14]. Some target proteins, such as G_sα and G_iα, are modified by both endogenous enzymes and bacterial toxins. This raises the question, how similar are these enzymes to the bacterial toxins? They may both share the same enzyme mechanism and substrates and yet one will work under the control of the cellular signalling pathways, while the other dramatically disrupts cellular signalling and is toxic to the cell. Such questions may only be addressed

when more structural information is available concerning endogenous enzymes, and this requires the isolation of these enzymes from cellular sources.

For cholera toxin [15] and pertussis toxin [16], it has been demonstrated that, in the absence of the protein target, the free amino acid may act as an acceptor for ADP-ribose. On the basis that endogenous enzymes may share the same enzyme mechanism as the bacterial toxins, this property was used to identify endogenous ADP-ribosyltransferases. The enzyme activity is detected simply by measuring the nicotinamide released in the presence of the amino acid acceptor using the NAD⁺ glycohydrolase (NAD⁺ase) assay [15]. Several arginine-specific ADP-ribosyltransferases have been purified on this basis [17–19]. Estimates *in vivo* of the amount of ADP-ribosylated protein in cells suggest that proteins modified through cysteine residues are as abundant as arginine- and carboxylate-modified residues [20]. However, only one NAD⁺:cysteine ADP-ribosyltransferase has been purified [21]. In this paper we describe the purification of a cysteine-dependent NAD⁺ase from bovine erythrocytes and demonstrate that it has a novel mono-ADP-ribosyltransferase activity.

MATERIALS AND METHODS

Materials

Purified pertussis toxin was a kind gift from Dr. L. Irons (PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, U.K.). Cholera toxin and pig brain NAD⁺ase were purchased from Sigma (Poole, Dorset, U.K.); NAD⁺ and ADP-ribose were from Boehringer Mannheim (Lewes, Sussex, U.K.).

Abbreviations used: G_i, subunit of inhibitory G-protein; G_s, subunit of stimulatory G-protein; NAD⁺ase, NAD⁺ glycohydrolase (EC 3.2.2.5); NEM, *N*-ethylmaleimide.

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Matrex Orange dye affinity chromatography resin was purchased from Amicon (Stonehouse, Gloucestershire, U.K.), cyanogen bromide-activated Sepharose from Pharmacia (Milton Keynes, Hertfordshire, U.K.), and Spherisorb S5-SAX HPLC column from Anachem (Luton, Bedfordshire, U.K.). Radiochemicals, [*nicotinamide*-³H]NAD⁺ and [*adenylate*-³²P]NAD⁺ were purchased from Amersham (Amersham, Buckinghamshire, U.K.). All other materials used were of the highest grade available from commercial sources.

NAD⁺ase assay

A modification of the method of Moss and Vaughan [15] was followed to detect the release of nicotinamide from NAD⁺. A typical assay contained 50 mM phosphate buffer, pH 7.0, and 100 μ M [*nicotinamide*-³H]NAD⁺ (1 mCi/mmol) in a total reaction volume of 150 μ l. Control incubations, without any enzyme added, were run for each reaction condition to account for non-enzymic hydrolysis of NAD⁺ (typically 0.01 % of total NAD⁺). The reaction was started by addition of the substrate NAD⁺, incubated in a water bath at 30 °C for 15 min, and stopped by addition of 0.85 ml of ice-cold water. A 0.9 ml aliquot was removed and applied to 0.5 ml of washed Dowex AG1X anion-exchange resin, and the unbound material containing [³H]nicotinamide was collected. [³H]Nicotinamide was washed off the column completely after application of two 0.5 ml portions of water. Eluate (0.5 ml) was removed and the amount of ³H label was measured by scintillation counting. Each sample was measured in duplicate and the reaction conditions were set such that a maximum of 20 % NAD⁺ was hydrolysed during the time course of the experiment. The cysteine-dependent activity was calculated as the difference in the amount of label released between incubations with and without 100 mM cysteine for enzyme and blank. In the absence of enzyme, background hydrolysis increased to 0.05 % of total NAD⁺ in the presence of cysteine.

Purification of cysteine-dependent NAD⁺ase

Fresh bovine blood (1 l) was collected from the slaughterhouse, and 3.5 % (w/v) sodium citrate was added to prevent coagulation. The erythrocytes were prepared according to the method of Kaslow et al. [22], and lysed in hypotonic buffer, 10 mM potassium phosphate, pH 7.8, containing 0.1 mM PMSF and 0.1 % (v/v) 2-mercaptoethanol. Solid (NH₄)₂SO₄ was added to the lysate to give a 40 % saturated solution, and proteins were allowed to precipitate at 4 °C for 3 h. The precipitated protein was collected by centrifugation (14000 g, 30 min). The enzyme activity was stable when stored as an (NH₄)₂SO₄ precipitate below 4 °C. One-sixth of the precipitate was processed through the remaining purification steps. The precipitated protein was solubilized in buffer A [50 mM potassium phosphate/30 % ethylene glycol/0.1 mM PMSF/0.1 mM EDTA/0.1 mM benzamidine (pH 7.0)] and dialysed to remove salts and traces of 2-mercaptoethanol. The sample was applied slowly to a cysteine-Sepharose column (bed volume 30 ml) at a flow rate of 0.2 ml/min. The cysteine-Sepharose column was prepared by reacting free cysteine with cyanogen bromide-activated Sepharose, following the manufacturer's recommended protocol. The amount of ligand bound to the column was estimated to be 2.5 μ mol/ml, using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) [23] to detect the amount of free thiol bound to the support. Unbound proteins were washed off the column with ten column volumes of buffer A. Bound protein was eluted with successive washes with buffer B [buffer A containing 0.1 % (v/v) 2-mercaptoethanol], buffer B with 0.1 M NaCl and buffer

B with 0.5 M NaCl. Cysteine-dependent activity was eluted in buffer B with 0.1 M NaCl, and was immediately mixed with 2 ml of Matrex Orange resin and rotated gently for 1 h before packing the resin into a 1-cm-diameter column. The gel was washed with ten column volumes of buffer B/0.1 M NaCl and the bound activity was eluted in buffer A/0.5 M NaCl. This fraction was stored immediately at -16 °C, and the activity was stable for up to three months in the absence of thiols.

ADP-ribosylation of membrane proteins

Preparation of ghost membranes

Washed bovine erythrocytes were prepared as described above. Following lysis of the cells with hypotonic buffer, the ghosts and unbroken cells were collected by centrifugation (14000 g, 25 min) and washed with hypotonic buffer three times, after which no more haemoglobin was detectable in the supernatant. The ghost membranes were incubated with hypotonic buffer containing 0.1 mM EDTA (inverting buffer) for 15 min at 30 °C to invert them.

ADP-ribosylation incubation

The optimized ADP-ribosylation conditions characterized for pertussis toxin by Ribeiro-Neto et al. [24] were followed. A typical ADP-ribosylation reaction contained 20–40 μ g of bovine erythrocyte membrane proteins, 5 mM ATP, 0.1 mM GTP, 0.1 mM PMSF, 2 mM EDTA, 50 mM sodium phosphate, pH 7.0, and 0–10 μ g of activated pertussis toxin (activated according to the conditions described by Kaslow et al. [25]) or 0–200 ng of purified cysteine-dependent NAD⁺ase in a reaction volume of 45 μ l. A control assay reaction was performed containing all of the above ingredients except pertussis toxin or endogenous enzyme. The reaction was started by the addition of 2 μ M [*adenylate*-³²P]NAD⁺ (20–30 mCi/mmol) and incubated at 30 °C for 30 min. The reaction was stopped by the addition of an equal volume of 10 % ice-cold trichloroacetic acid, and the proteins were allowed to precipitate on ice for 10 min. The precipitated membrane proteins were collected by centrifugation (13000 g, 5 min) and were washed three times with 0.1 % trichloroacetic acid to remove excess NAD⁺. ADP-ribosylation of bovine erythrocyte ghosts by 5 μ g of cholera toxin was achieved using a modification of the above procedure. MgCl₂ (10 mM) was added to promote the activation of G_s α , the substrate for cholera toxin. Magnesium is not included in pertussis toxin incubations because it inhibits ADP-ribosylation of G_i α [26]. After precipitation and washing, the membrane proteins were solubilized in 2 % (w/v) SDS/50 mM Tris/HCl (pH 6.8). The proteins were separated on an SDS/polyacrylamide (10 %) gel and were stained with Coomassie Brilliant Blue. The radiolabelled proteins were identified by autoradiography. The gel was dried and then exposed to Hyperfilm-MP (Amersham) using Kodak screen intensifiers for 24–48 h at -70 °C. The amount of label per lane was quantified by scanning the density of the band using a scanning densitometer (Joyce/Loebl). To account for any uneven protein loading, the density of erythrocyte band V, revealed by Coomassie Brilliant Blue staining, was measured.

Reaction of free ADP-ribose

Preparation of free [³²P]ADP-ribose

[*Adenylate*-³²P]ADP-ribose was prepared from [*adenylate*-³²P]NAD⁺ by incubation with 10 μ g of pig brain NAD⁺ase in 50 mM potassium phosphate buffer, pH 7.2, at 30 °C for 30 min.

Table 1 Purification of cysteine-dependent NAD⁺ase

Activity was determined using the NAD⁺ase assay as described in the Materials and methods section. Protein concentration was estimated using Bradford reagent with BSA (Sigma) as a standard [36].

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Fold purification	Yield (%)
Lysate	16250	690	0.04	1	100
40% (Na ₄) ₂ SO ₄	120	400	3	75	58
Cysteine-Sepharose	0.6	152	250	6250	22
Matrex Orange	0.06	116	1900	47500	16

Figure 1 Silver-stained gel of final purified product

Material from the final stage of the purification (0.5 µg), which had cysteine-dependent NAD⁺ase activity, was applied to an SDS/polyacrylamide (10%) gel. The sample was run under reducing conditions and was stained with silver [37]. An *M_r* of 45 000 was estimated by comparison with known molecular-mass standards (66 000–14 300; Sigma) as indicated.

The protein was removed by filtration through a membrane microconcentrator of *M_r* cut-off 10 000. ADP-ribose was separated from NAD⁺ on application of the filtrate to a Spherisorb S5-SAX HPLC column (50 mm × 4.6 mm; Anachem), which had been equilibrated in 100 mM potassium phosphate buffer, pH 4.5 [3]. The products were separated on the column by isocratic elution in 100 mM potassium phosphate, pH 4.5, and monitored using a UV detector (254 nm).

ADP-ribosylation reaction

The ADP-ribosylation reaction was set up as described above in the absence of added enzyme. [³²P]ADP-ribose (0.4 µM) was substituted for [³²P]NAD⁺ (1.0 µM) in the assay and incubated with the erythrocyte ghost membranes at 30 °C for 15 min. The labelling observed was compared with that seen with NAD⁺ in the presence and absence of added enzyme.

Identification of the amino acid linkage

Treatment of ghost membranes with *N*-ethylmaleimide (NEM)

Prepared bovine erythrocyte membranes were washed three times with 50 mM potassium phosphate buffer (pH 7.2)/0.1 mM PMSF/0.1 mM EDTA to remove excess 2-mercaptoethanol. NEM (1 mM) (freshly prepared) was then added to the membranes which were then incubated at 30 °C for 30 min. Excess NEM was removed by washing the membranes three times with inverting buffer. A control membrane preparation was treated as above, except that NEM was not added.

Release of label

The sensitivity of the ADP-ribosyl-protein linkage to cleavage by neutral hydroxylamine or mercuric ion was tested. After precipitation and washing of the membrane proteins the sample was divided into three equal portions and incubated (with shaking) overnight at 4 °C with 0.5 M neutral hydroxylamine/0.5 M NaCl (pH 7.4), 10 mM mercuric acetate (pH 3.0) or 50 mM potassium phosphate buffer/0.5 M NaCl (pH 7.4). The precipitate was not solubilized under these conditions, but remained as a fine white precipitate in suspension. The precipitate was collected by centrifugation (13 000 *g*, 5 min) and the proteins were then solubilized in SDS/sample buffer and separated by SDS/PAGE (10% gel), stained, destained and autoradiographed.

Detection of product released by mercuric ion

To detect the type of label released by mercuric acetate it was necessary to remove excess NAD⁺ efficiently. This was achieved by first separating the products on SDS/polyacrylamide (10%) gels, and then electro-eluting the labelled protein (3 W, constant power, 3 h) using ISCO electro-elution apparatus. The eluted protein was then treated with 10 mM mercuric acetate at 30 °C for 30 min. The protein was precipitated by adding 5% (v/v) perchloric acid. The supernatant was neutralized by addition of potassium hydroxide (at 4 °C), and the insoluble salt (potassium perchlorate) formed was removed by centrifugation (13 000 *g*, 5 min). The sample was diluted 2-fold with 100 mM potassium phosphate, pH 4.5, before applying it to a S5-SAX HPLC column and separating the products as described above. Fractions of 0.2 ml were collected and 4 ml of scintillant (HiSafe; Pharmacia) was added. The samples were counted for radioactivity for 10 min using a Packard 1900CA scintillation counter. The elution profiles were compared to NAD⁺ and ADP-ribose standards run under comparable conditions.

Figure 2 Saturation kinetics observed for cysteine-dependent NADase

The rates of nicotinamide released in the presence of (a) 100 mM cysteine and 0–250 μ M NAD⁺ and (b) 0–200 mM cysteine and 100 μ M NAD⁺ are shown. Each assay was performed in duplicate and the data presented are representative of three separate experiments. The data were fitted to the Michaelis–Menten equation and gave K_m 8 μ M for NAD⁺ and 4.0 mM for cysteine.

RESULTS

A cysteine-dependent NADase activity was found in freshly prepared bovine erythrocyte lysate and was purified 47000-fold following a three-step purification procedure. A typical purification is shown in Table 1. The cysteine-dependent NADase activity was measured as the difference between the nicotinamide released from NAD⁺ in the presence and absence of cysteine. In crude samples many enzymes were present that contributed to NAD⁺ hydrolysis, including NADases that may be either activated or inhibited by cysteine and other ADP-ribosyltransferases. The level of non-specific NADase activity in the lysate was 60 pmol/min per mg of protein, similar to the levels of NADase activity reported in the human erythrocyte [27]. Detection of a cysteine-dependent activity against these high levels of background activity was only possible in fresh lysates. The non-specific, contaminating NADases were removed by the cysteine-Sepharose affinity column, and a yield of 16% of the activity was obtained after binding to Matrex Orange. The protein had a specific activity of 1900 nmol nicotinamide released/min per mg, and was inactive in the absence of cysteine. Figure 1 shows that the purified product separated by SDS/PAGE (10% gel) appeared as a single band, after silver staining, with an apparent M_r of 45000. The presence of 30% (v/v) ethylene glycol and protease inhibitors in the buffers was essential for maintaining the activity of the enzyme.

The enzyme was purified on the basis that, like pertussis toxin, the enzyme would be able to use cysteine as an acceptor for

Table 2 Substrates for cysteine-dependent NADase

Activity was measured using the NADase assay as described in the Materials and methods section. Each assay contained 100 μ M NAD⁺, 100 mM test substrate and 100 ng of purified enzyme. Each assay was performed in duplicate and the results are representative of three separate experiments. The limit of detection for the assay was 10 pmol/min.

Substrate	Activity (pmol/min)
Cysteine	300
S-carboxymethylcysteine	< 10
Arginine methyl ester	< 10
Glycine	< 10
Dithiothreitol	87

Figure 3 ADP-ribosylation of bovine erythrocyte membranes

ADP-ribosylation of 40 μ g of bovine erythrocyte membrane proteins was performed as described in the Materials and methods section. Autoradiographs of the radiolabelled membrane proteins, separated on SDS-polyacrylamide (10%) gels after incubations containing (a) membranes only, (b) 0.1 μ g of cysteine-dependent NADase, (c) 2 μ g of pertussis toxin and (d) 5 μ g of cholera toxin, are shown. The labelling observed from erythrocyte membranes that were washed with a high-salt buffer [50 mM phosphate buffer (pH 7.2)/200 mM NaCl/0.1 mM PMSF/0.1 mM EDTA/0.1% (v/v) 2-mercaptoethanol] are shown in lanes (e) without and (f) with cysteine-dependent NADase. Positions of molecular-mass standards are indicated on the right.

ADP-ribose but no other amino acid. The substrate specificity of the purified enzyme was tested using the NADase assay. The dose–response curves observed for NAD⁺ and cysteine are shown in Figure 2. The data were fitted to the Michaelis–Menten equation using non-linear regression curve fitting. An apparent K_m of 8 μ M for NAD⁺ was calculated and 4.0 mM for cysteine. The specificity of cysteine as a substrate was tested by substituting for it with 100 mM S-carboxymethylcysteine, arginine methyl ester, glycine or dithiothreitol in the assay. The activity observed is shown in Table 2. A free thiol group appeared to be essential for activity to be observed. Only dithiothreitol showed any activity, whereas other amino acids or cysteine derivatives in

Figure 4 Dose-response curve

Erythrocyte membrane proteins (15 μ g) were incubated with 0–200 ng of cysteine-dependent NADase for 30 min at 30 °C. The graph shows the amount of radiolabel incorporated into the protein of M_r 55000 in response to added enzyme. The density of labelling observed is expressed relative to the background labelling observed in the absence of enzyme (5000 arbitrary units). Positions of molecular-mass standards are indicated on the right.

which the thiol group is blocked by a methyl ester group did not. The lack of activity in the presence of 100 mM arginine methyl ester confirmed that the purified protein was free from any NAD⁺:arginine ADP-ribosyltransferase. To investigate the ability of the purified enzyme to ADP-ribosylate protein substrates it was incubated with washed inside-out erythrocyte membranes and [³²P]NAD⁺. The labelling observed was compared with that seen with cholera and pertussis toxin (Figure 3). In the absence of enzyme or toxin, labelling by [³²P]NAD⁺ of proteins of M_r 55000, 60000 and a doublet of high molecular mass was observed. The intensity of all of these bands increased on addition of the cysteine-dependent NADase and a very faint band appeared at M_r 29000. The dose-dependent increase of labelling of the major bands at M_r 60000 and 55000 is shown in Figure 4. Pertussis toxin also caused an increase of labelling at M_r 55000, as well as labelling of a protein of M_r 41000. Incubations containing cholera toxin showed labelling of several proteins with M_r s 66000, 45000 and 42000, and did not cause increased labelling of M_r 55000. The protein of M_r 41000 labelled by pertussis toxin and the proteins of M_r s 45000 and 42000 labelled by cholera toxin were confirmed as $G_{i\alpha}$ or $G_{s\alpha}$ by Western blotting using anti-peptide antibodies to $G_{i\alpha}$ or $G_{s\alpha}$. The enzyme-labelled proteins were not recognized on Western blots by these anti-peptide antibodies, and it is therefore unlikely that any of the target proteins were modified G-proteins. After treatment of the erythrocyte membranes with a salt wash [50 mM phosphate buffer (pH 7.2)/200 mM NaCl/0.1 mM PMSF/0.1 mM EDTA/0.1% (v/v) 2-mercaptoethanol], the major band at M_r 55000 was the only one seen to be labelled (Figure 3, lanes e and f). Such treatment, however, has not reduced the intensity of background labelling of this protein.

ADP-ribose is a highly reactive species, and has been shown to react with proteins directly, modifying lysine [12], arginine [28] and cysteine [14] residues. The possibility that the ADP-ribosylation observed was due to increased levels of free ADP-ribose, generated by the NADase activity of the enzyme added, was tested. [³²P]ADP-ribose was generated from [³²P]NAD⁺ by NADase from pig brain, and was purified by ion-exchange

Table 3 ADP-ribosyl linkage tests

The amount of labelling associated with a protein of M_r 55000 separated on SDS/polyacrylamide (10%) gels was quantified by scanning densitometry of an autoradiograph, and uneven protein loading was accounted for by comparison of the density of Coomassie Brilliant Blue staining of erythrocyte band V. The amount of labelling detected following the above treatments was compared with untreated ADP-ribosylated membrane proteins (20000 arbitrary density units in the absence of cysteine-dependent NADase and 30000 arbitrary density units in the presence of enzyme). The data shown are representative of labelling observed from three separate experiments.

Treatment	Label remaining after treatment (%) without cysteine-dependent NADase	With cysteine-dependent NADase
<i>N</i> -ethylmaleimide	90	15
Neutral hydroxylamine	100	80
Mercuric acetate	90	60
0.5 M NaCl, pH 7.4	100	100

HPLC. Free ADP-ribose was added to the incubation at a concentration equivalent to 45% of the total NAD⁺ in the assay having been hydrolysed. This level was much higher than may be expected to be generated by the cysteine-dependent NADase during the time course of the assay, given that the concentration of NAD⁺ (10 μ M) and cysteine (not added) were sub-saturating; however, NADases associated with the erythrocyte membrane [29] may have been present and could account for the background labelling. No labelling of any membrane protein was observed in the presence of free [³²P]ADP-ribose. This suggests that the labelling observed in the presence or absence of the cysteine-dependent NADase was enzyme mediated and not a result of increased levels of ADP-ribose that were then able to react with membrane proteins.

Since free cysteine appeared to be a substrate for the purified enzyme in the NADase assay it seemed likely that the protein labelling observed was through modification of a cysteine residue. The membrane proteins were pre-incubated with 1 mM NEM, in order to alkylate any available thiols, and the effect on labelling was investigated. As can be seen in Table 3, background labelling of the M_r 55000 membrane protein was unchanged following NEM treatment, whereas the enhanced labelling observed on addition of the cysteine-dependent NADase was completely inhibited. This suggests that the background labelling observed occurs either on the same protein but on a different residue, or on a different protein of similar molecular mass. Further evidence to support a cysteine-ADP-ribose modification was obtained by selective removal of the label by chemical treatment. The thio-glycosidic bond formed should be sensitive to nucleophilic attack by mercuric ion [30], but stable to neutral hydroxylamine [31]. The ADP-ribosylation reaction was stopped on addition of trichloroacetic acid, which precipitated the proteins. The precipitate was treated with either 0.5 M neutral hydroxylamine, 10 mM mercuric acetate or a control treatment of 50 mM potassium phosphate/0.5 M NaCl, and then solubilized in SDS/sample buffer and separated by SDS/PAGE (10% gels). The amount of label remaining associated with a M_r 55000 protein was determined by densitometry and is shown in Table 3. In samples incubated in the presence of enzyme, neutral hydroxylamine had no effect on the labelling, whereas mercuric acetate released 40% of the label, reducing the intensity of labelling to that observed in the absence of added enzyme. Neither treatment affected the background labelling. Under these same conditions, hydroxylamine completely removed cholera toxin ADP-

Figure 5 Detection of ^{32}P -labelled product by HPLC

Radiolabelled protein of M_r 55 000 was eluted from an SDS/polyacrylamide gel and treated with 10 mM mercuric acetate as described in the Materials and methods section. NAD^+ (100 μM) and ADP-ribose (100 μM) standards were added to the released radiolabel to act as internal controls, before application to S5-SAX ion-exchange HPLC. The column was equilibrated in 100 mM potassium phosphate buffer, pH 7.4, and NAD^+ and ADP-ribose were separated on this column by isocratic elution (1 ml/min) in this buffer. The standards were detected with a UV monitor (254 nm) (a), and the radiolabelled product was detected by scintillation counting for 10 min (b) of the 0.2 ml fractions collected from samples labelled in the presence (○) or absence (●) of cysteine-dependent NAD^+ ase.

ribosylation of G_α , and mercuric acetate completely removed pertussis toxin ADP-ribosylation of G_α (results not shown).

Finally, to confirm that the modified cysteine residue, and not some other adduct of NAD^+ , was ADP-ribosylated, the released label was separated by HPLC. First it was necessary to remove unincorporated radiolabel completely. This was achieved by separation of the proteins by SDS/PAGE. Protein of M_r 55 000, which had been labelled in the presence or absence of cysteine-dependent NAD^+ ase, was eluted from the gel and treated with 10 mM mercuric chloride. The released products were applied to an S5-SAX HPLC column equilibrated in 100 mM potassium phosphate, pH 4.5, and were eluted isocratically. Figure 5 shows the elution profile obtained from the released products compared with NAD^+ and ADP-ribose standards. No released product was detected for membranes incubated without the cysteine-dependent NAD^+ ase. However, a radiolabelled product was released from the M_r 55 000 protein incubated with cysteine-dependent NAD^+ ase, which had an elution time coincident with that of ADP-ribose run under identical conditions. These experiments

provide strong evidence that the cysteine-dependent NAD^+ ase activity purified could ADP-ribosylate a specific membrane target protein via a cysteine residue on that protein.

DISCUSSION

The NAD^+ ase activity may be considered as the half reaction of ADP-ribosyltransferases where water acts as the acceptor for ADP-ribose instead of the target protein. Free amino acids may also act as acceptors for ADP-ribose. These properties are exhibited by the bacterial toxin ADP-ribosyltransferases and have been used to select for novel ones, such as NAD^+ :arginine ADP-ribosyltransferases from turkey erythrocytes [17] and skeletal muscle [19] and NAD^+ :cysteine ADP-ribosyltransferase from human erythrocytes [21]. The enzyme purified in this study was much larger than the previously identified NAD^+ :cysteine ADP-ribosyltransferase, which had a M_r 28 000, similar in size to the S1 subunit of pertussis toxin. This suggests that, although similar strategies were followed to purify these enzymes, different products were obtained. The affinity of the purified enzyme for cysteine was much higher than that exhibited by pertussis toxin ($K_m = 100 \text{ mM}$) [16], but similar to that shown by the human enzyme ($K_m = 4.4 \text{ mM}$) [21].

Direct evidence that free cysteine is an acceptor for ADP-ribose has not been shown in these experiments. The increase in nicotinamide release observed in the presence of free thiol (cysteine or dithiothreitol) may result from activation of the enzyme, by reduction of a disulphide bond. It cannot be assumed that cysteine is an acceptor for ADP-ribose by comparison with pertussis toxin. Evidence has been reported that shows that the ADP-ribose-cysteine adduct previously identified [16] could be generated non-enzymically [32]. The ADP-ribose-cysteine adduct was shown to be ADP-ribosylthiazolidine by NMR, and postulated to be generated non-enzymically by the reaction of free ADP-ribose with the amine group of cysteine.

Increased labelling of several bovine erythrocyte membrane proteins was observed on addition of the cysteine-dependent NAD^+ ase. Only the major component of M_r 55 000 was stable to salt washing of the membranes, suggesting that the minor components observed are loosely associated and are not integral membrane proteins. Interestingly the background labelling of this protein was not significantly reduced by this treatment. This suggests that if the labelling is enzymic then the enzyme involved must be tightly associated with the membrane. It is therefore unlikely that the background activity is due to residual amounts of the cysteine-dependent NAD^+ ase, which is purified from the bovine erythrocyte cytosol.

There are three lines of evidence to suggest that a cysteine residue of the major target protein of M_r 55 000 was ADP-ribosylated. (1) The labelling was sensitive to pre-treatment with NEM. (2) The labelling was sensitive to mercuric chloride and insensitive to neutral hydroxylamine. (3) The label released by mercuric ion was shown by HPLC to have an elution time coincident with that of ADP-ribose. Mercuric acetate did not release the background labelling, further supporting the hypothesis that the background labelling observed is different to the labelling catalysed by cysteine-dependent NAD^+ ase. The background label was also stable to neutral hydroxylamine. The possibility that the background labelling is enzyme-mediated ADP-ribosylation of some other amino acid residue, e.g. lysine, remains. The background labelling is not a result of reaction of free ADP-ribose with reactive residues, but the possibility of an NAD^+ adduct cannot be dismissed. It has also not been determined whether the background labelling and the enzyme-catalysed reaction are modifying the same protein or two different

proteins of similar molecular mass. Transducin is an example of a protein that may be ADP-ribosylated at more than one site [3]. The M_r 55000 protein is a novel target protein that has not been reported previously. The previous NAD⁺:cysteine ADP-ribosyltransferase isolated from human erythrocytes ADP-ribosylated the same target protein as pertussis toxin, $G_i\alpha$.

The relative abundances of ADP-ribose-cysteine- and ADP-ribose-arginine-modified proteins in whole cells are similar [4]. Many of the modified cysteine proteins identified to date, for example β actin [14], have been shown to be generated non-enzymically. Some non-enzymic modifications occur as a result of a direct stimulus, for example stimulation of labelling of an active site thiol of GAPDH in response to nitric oxide. This activity was first reported as a novel ADP-ribosyltransferase activity [33] and subsequently shown to occur non-enzymically by activation of the free thiol with nitric oxide to form an *S*-nitrosyl group and reaction with NAD⁺ directly [34]. The possibility that the labelling observed was a result of free ADP-ribose reacting with a susceptible thiol group on the protein target was ruled out by the absence of labelling of any proteins following incubation of membrane proteins with [³²P]ADP-ribose. Thus this is the first demonstration of a novel NAD⁺:cysteine ADP-ribosyltransferase activity modifying an M_r 55000 substrate. The purification strategy employed has selected for the amino acid specificity of the enzyme but not whole protein target specificity. Similar results were observed for NAD⁺:arginine ADP-ribosyltransferases purified on the basis of their ability to use small guanidine-containing compounds as acceptors for ADP-ribose. The enzymes purified were found not to ADP-ribosylate $G_s\alpha$ as might have been expected, but were found to specifically modify the M_r 105000 subunit of Ca²⁺-dependent ATPase [35].

The results presented here provide further evidence that the ADP-ribosyltransferase reaction is not restricted to the bacterial toxins. Endogenous enzymes exist that have similar amino acid specificity but have different protein targets. It appears that endogenous NAD⁺:cysteine ADP-ribosyltransferases exist even in the simplest of cells, such as the erythrocyte, supporting the hypothesis that endogenous ADP-ribosyltransferases have a ubiquitous role in cellular signalling pathways. To understand the role of this enzyme in cellular metabolism will require further characterization not only of the enzyme but also of the protein target.

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