Biochem. J. (1983) **212**, 459–464
Printed in Great Britain

NAD$^+$ glycohydrolase, an ecto-enzyme of calf spleen cells

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(*Received 26 November 1982/Accepted 18 February 1983*)

By using a sensitive fluorimetric assay of NAD$^+$ glycohydrolase (EC 3.2.2.6), we showed that calf spleen cells are able to hydrolyse 1,N^6-etheno-NAD$^+$ given in the medium. The observed rates of substrate hydrolysis and product accumulation in the medium are equivalent. Moreover, the splenocytes are able to cleave the nicotinamide–ribose bond of a water-soluble polymer of NAD$^+$, and their NAD$^+$ glycohydrolase activity is fully inhibited by a high-molecular-weight Blue Dextran. Selective permeation of the cellular membrane digitonin revealed an intracellular pool of NAD$^+$ glycohydrolase, which accounts for 25% of the total activity. We conclude that NAD$^+$ glycohydrolase associated with the splenocytes has the characteristics of an ecto-enzyme.

Mammalian NAD$^+$ glycohydrolase (EC 3.2.2.6) is a membrane-bound enzyme which catalyses the hydrolytic cleavage of NAD$^+$ at the nicotinamide–ribose bond. The highest enzyme activity was classically found associated with microsomal fractions in a variety of tissues (Jacobson & Kaplan, 1957; Artman *et al.*, 1964; Bock *et al.*, 1968). Earlier studies have indicated that, e.g. in liver, NAD$^+$ glycohydrolase is a constituent of endoplasmic reticulum, plasma membranes (Bock *et al.*, 1971; Diaugustine *et al.*, 1978), nuclear envelopes (Green & Dobrjansky, 1972; Fukushima *et al.*, 1976; Tamulevicius *et al.*, 1979) and secondary lysosomes (Mellors *et al.*, 1975). The physiological role of this enzyme remains elusive; it was thought to control intracellular concentrations of NAD$^+$ (Kaplan, 1966; Johnson, 1980), but its occurrence at high concentrations in certain tissues was difficult to reconcile with the known turnover rates of the coenzyme *in vivo* (Bock *et al.*, 1968; Clark & Pinder, 1969; Bernofsky & Pankov, 1973).

In this context we have previously shown that NAD$^+$ glycohydrolase in calf spleen, although microsomal, was in fact predominantly if not exclusively associated with vesicles derived from plasma membranes (Muller & Schuber, 1980). Moreover, topological studies revealed that the active site of the enzyme was on the exterior side of these vesicles, suggesting that NAD$^+$ glycohydrolase might be an ecto-enzyme (Muller & Schuber, 1980), i.e. a plasma-membrane-associated enzyme which has its active site oriented to the external surface of the cell (De Pierre & Karnovsky, 1973).

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In the present study we provide evidence that in intact calf splenocytes, NAD$^+$ glycohydrolase fulfils the criteria commonly used to identify an ecto-enzyme. This finding, which is consistent with some earlier observations on NAD$^+$ cleavage by intact cells (Bekierkunst, 1967; Bock *et al.*, 1968; Liersch *et al.*, 1971; Alivisatos *et al.*, 1974; Pekala & Anderson, 1978; Artman & Seeley, 1979), has important consequences on determining the function of this enzyme and on its relationship to other NAD$^+$-catabolizing enzymes, e.g. poly(ADP-ribose) polymerase.

Materials and methods

Materials

1,N^6-Etheno-NAD$^+$ and 1,N^6-etheno-pyridine–adenine dinucleotide were synthesized from NAD$^+$ and pyridine–adenine dinucleotide (PyAD$^+$) (Kaplan & Ciotti, 1956) respectively, as described by Barrio *et al.* (1972) and Schlessinger & Levitzki (1974). Blue Dextran (M_r 40000) was prepared as described previously (Muller & Schuber, 1980) and soluble poly-N^6-(acryloxy-2-hydroxypropyl)-NAD$^+$ (M_r 20000) was kindly given by Dr. S. Sicsic (Cercoa, Thiais, France). Hanks balanced salt solution (Ca^{2+}- and Mg^{2+}-free), Trypan Blue and newborn-calf serum were purchased from Gibco. All other biochemicals or products were obtained from Sigma.

Preparation of splenocytes

Spleen slices were removed aseptically within minutes after the death of the animal and were

maintained in a heparinized (5 units/ml) Hanks medium at 20°C. Less than 15 min later a cell suspension was prepared by gently teasing the spleen pulp through an 80 μm stainless-steel grid partially immersed in the same medium. After collection of the cells by low-speed centrifugation (200 **g** for 15 min), the erythrocytes were lysed with an NH_4Cl solution (Boyle, 1968). After careful elimination of the erythrocytes, the splenocytes were resuspended in Hanks medium (3×10^7 cells/ml) containing 20% (v/v) heat-inactivated newborn-calf serum and stored in a silicone-coated spinner flask in a humidified incubator, at 37°C. The cells were counted with a haemocytometer and their viability was estimated by the Trypan Blue exclusion test and by phase-contrast inspection. Under our experimental conditions the average viability was about 95%; it remained almost constant over at least 4 h, and no contamination could be detected.

NAD⁺ glycohydrolase assays

NAD^+ glycohydrolase activity was determined routinely by the cyanide-addition assay (Schuber & Travo, 1976). A fluorimetric assay (Lee & Everse, 1973; Pekala & Anderson, 1978), which allows monitoring of reaction progress, with $1,N^6$-etheno-NAD^+ as substrate, was also used. The kinetic parameters of the hydrolysis of NAD^+ and of its fluorescent analogue are very similar, e.g. for NAD^+ $K_m = 66\,\mu$M and $V = 0.49\,\mu$mol/min per mg of protein, and for $1,N^6$-etheno-NAD^+ $K_m = 42\,\mu$M and $V = 0.53\,\mu$mol/min per mg of protein, for the membrane-bound form (Travo *et al.*, 1979) of calf spleen NAD^+ glycohydrolase. The reaction mixtures contained 60 μM-$1,N^6$-etheno-NAD^+ (assay A) or 60 μM-$1,N^6$-etheno-NAD^+ and 550 μM-NAD^+ (assay B; saturating conditions), a portion of cell suspension and phosphate-buffered saline (6.48 mM-Na_2HPO_4, 1.47 mM-KH_2PO_4, 2.68 mM-KCl, 136.75 mM-NaCl; pH 7.4), at 25°C (final volume 2 ml). Quantitative estimates of rates can be obtained by use of the known quantum-yield ratio (products to substrate), or by addition, to the reaction mixtures, of known amounts of internal standards (e.g. $1,N^6$-etheno-ADP-ribose or $1,N^6$-etheno-AMP). A unit of NAD^+ glycohydrolase activity was defined as the amount of enzyme that catalyses the cleavage of 1 μmol of $1,N^6$-etheno-NAD^+/min under the saturating conditions.

Since the increase in fluorescence after the hydrolytic cleavage of $1,N^6$-etheno-NAD^+ could also be due to the action of a nucleotide pyrophosphatase (EC 3.6.1.9), we have routinely checked that we were only measuring NAD^+ glycohydrolase activity. For such a control we used $1,N^6$-etheno-$PyAD^+$, which is not hydrolysed by NAD^+ glycohydrolase (Schuber *et al.*, 1979), but under optimal conditions (alkaline pH, bivalent cations) is an excellent substrate for nucleotide pyrophosphatase (C. Muller, unpublished work). Under the conditions used for the NAD^+ glycohydrolase assay, almost no increase in fluoresence owing to hydrolysis of $1,N^6$-etheno-$PyAD^+$ was observed (less than 2% of the fluorescence increase observed with $1,N^6$-etheno-NAD^+).

Analysis of the reaction products was performed by high-performance liquid chromatography [conditions: 25 cm × 4.6 mm Ultrasphere ODS Column (Beckman) with mobile phase 89% water, 11% acetonitrile; isocratic].

Results

NAD⁺ glycohydrolase activity from whole splenocytes

A suspension of calf splenocytes was prepared, and the viability of the cells was assessed by their ability to exclude Trypan Blue and by phase-contrast-microscopic examination. The morphology of the cells appeared normal and the average cell viability, which remained high throughout the experiments, approached 95%.

The splenocytes were incubated in an iso-osmotic medium and the NAD^+ glycohydrolase activity was measured. Intact cells were able to hydrolyse NAD^+ (cyanide-addition assay) and $1,N^6$-etheno-NAD^+ as measured by the increase of fluorescence at 410 nm. Under saturating concentrations of substrate, NAD^+ glycohydrolase activity was proportional to cell number up to 10^7 cells/ml and the average activity was 2.15 ± 0.30 units/10^9 cells. NAD^+ glycohydrolase activity determined with splenocytes over the NAD^+ concentration range 15–600 μM showed classical saturation kinetics, and analysis of the data by a Lineweaver–Burk plot gave a K_m of 59 μM. In order to evaluate whether the observed NAD^+ glycohydrolase activity was associated with the cells or with the medium (damaged or broken cells, culture medium), the enzyme activity was determined in a sample of the supernatant obtained after collecting the cells from a suspension by low-speed centrifugation (200 **g** for 15 min). Virtually all NAD^+ glycohydrolase activity was found to be pelletable.

An important characteristic of reactions catalysed by ecto-enzymes is that the products are found in the external medium. An experiment was conducted to test this point. The kinetics of $1,N^6$-etheno-NAD^+ hydrolysis catalysed by intact cells, as measured by increase in fluorescence, were recorded. In parallel, runs of similar reaction mixtures were stopped at different times by addition of isonicotinic acid hydrazide (Zatman *et al.*, 1954); the fluorescence of the medium was measured after separation from the cells by filtration (the appearance in the medium of $1,N^6$-etheno-ADP-ribose, the

reaction product, was assessed by high-performance liquid chromatography). As shown in Fig. 1, the rate of 1,N^6-etheno-NAD+ hydrolysis by intact splenocytes and the rate of product appearance in the medium are in excellent agreement.

Effect of cell permeabilization

Mechanical disruption of the cells by use of a Dounce homogenizer (assessed by microscopic observation) did not markedly increase the NAD+ glycohydrolase activity of the calf splenocyte suspension. The cells were also permeabilized with increasing concentrations of digitonin. Their leakiness was monitored by Trypan Blue uptake and by the appearance of lactate dehydrogenase in the medium. This method, which requires very low concentrations of the permeabilizing agent (Fiskum *et al.*, 1980), because of the interaction of digitonin with cholesterol (Scallen & Dietert, 1969), affects mainly the permeability of plasma membranes (Fiskum *et al.*, 1980; Becker *et al.*, 1980). Fig. 2 illustrates the effect of digitonin on lactate dehydrogenase release from splenocytes and on NAD+ glycohydrolase activity. A small increase in NAD+ glycohydrolase activity was detected when the cells were permeabilized. (Within the detergent concentration range used for this study, all NAD+ glycohydrolase was found to remain membrane-bound.) It is noteworthy that the activity of membrane-bound NAD+ glycohydrolase, e.g. associated with microsomal fractions, is not affected by modifications of lipid–protein interactions induced by detergents (Muller & Schuber, 1980; Schuber *et al.*, 1980). It appears therefore that digitonin reveals an internal pool of NAD+ glycohydrolase, about 25% of total activity, which is inaccessible to extracellular NAD+ in intact cells. The use of Emulphogene BC-720, a non-ionic detergent (Helenius & Simons, 1975), at a concentration of 0.1% (w/v) also yielded a similar increase in NAD+ glycohydrolase activity (+35%) in the treated splenocytes. This detergent, which lacks the specificity of digitonin, should allow access to most compartments of the cell.

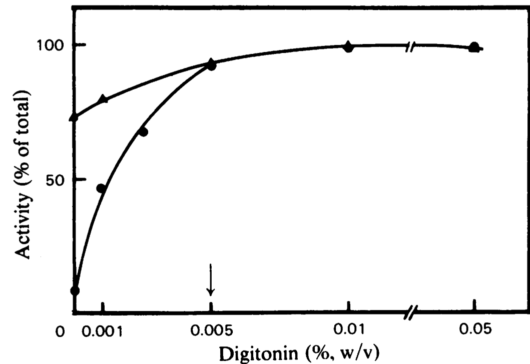

Fig. 2. *Effect of digitonin on the activity of NAD+ glycohydrolase and on the release of lactate dehydrogenase from splenocytes*

The cells (2.5 x 10^6 cells/assay) were incubated for 15 min at 25°C in phosphate-buffered saline in the presence of various concentrations of digitonin and were assayed for NAD+ glycohydrolase activity (▲) (assay B). In parallel runs lactate dehydrogenase activity (●) was measured in samples (20 μl) of the medium after separation of the cells. The activities are expressed as a percentage of total activity obtained at high detergent concentrations (the arrow corresponds to the digitonin concentration that gave maximal Trypan Blue uptake).

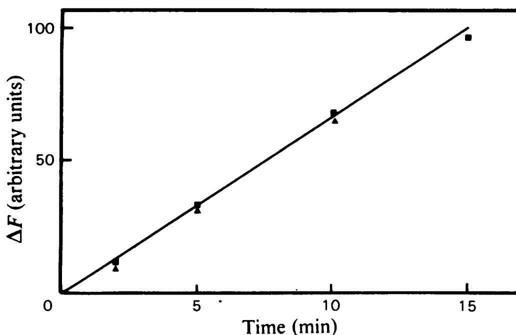

Fig. 1. *Comparative rates of NAD+ hydrolysis catalysed by splenocytes and of product appearance in the medium*

Calf spleen cells (3.5 x 10^6 cells/assay) were incubated with 60 μM-1, N^6-etheno-NAD+, at 25°C, in phosphate-buffered saline (final volume 2 ml). The increase in fluorescence at 410 nm (excitation 300 nm) as a function of time was recorded (■). In parallel runs, the reaction catalysed by cell-associated NAD+ glycohydrolase was stopped by addition of isonicotinic acid hydrazide (final concn. 1 mM), at the times indicated. The fluorescence of the medium (▲) was measured after filtration of the cells on Millipore filters. ΔF represents the difference in fluorescence of the medium at the given times and zero time.

Studies with non-permeant substrates and inhibitors

In order to substantiate further the localization of the NAD+ glycohydrolase active site on the outer surface of calf splenocytes, we have probed the activity of the enzyme with non-penetrating substrates and inhibitors. Blue Dextrans are very potent competitive inhibitors of the soluble and membrane-bound forms of the enzyme (Muller & Schuber, 1980; Schuber & Pascal, 1977). Fig. 3 shows that NAD+ glycohydrolase from intact splenocytes is efficiently inhibited (I_{50}, the con-

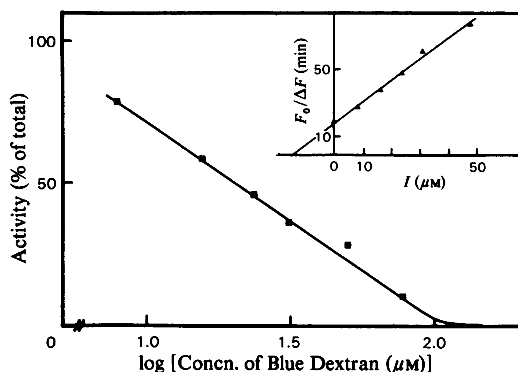

Fig. 3. *Inhibition of splenocyte-associated NAD$^+$ glycohydrolase by Blue Dextran*

Initial rates of 1,N^6-etheno-NAD$^+$ hydrolysis (assay B) catalysed by calf spleen cells (2.5×10^6 cells/assay) were determined at 25°C in the presence of various concentrations of Blue Dextran (M_r 40000). The results are expressed as percentages of the rate in the absence of inhibitor. Inset: reciprocal plot of initial rates versus inhibitor concentration, I (F_0, initial fluorescence; ΔF, fluorescence change as function of time). The concentration of inhibitor is expressed in terms of its chromophore.

centration of inhibitor required to decrease the reaction velocity by 50%, is 20μM) by a Blue Dextran, a highly negatively charged molecule, with an average M_r of 40000.

We have also tested a water-soluble polymer of NAD$^+$, i.e. poly-N^6-(acryloxy-2-hydroxypropyl)-NAD$^+$, which is active as a coenzyme for several dehydrogenases (Le Goffic *et al.*, 1980), and whose average M_r was 20000. Similarly to our previous findings with microsomal NAD$^+$ glycohydrolase (Muller & Schuber, 1980), this polymer of NAD$^+$ was a good substrate of intact splenocytes (results not shown).

Cell-membrane-impermeant reagents such as diazosulphanilic acid (De Pierre & Karnovsky, 1974) have also been used in order to identify ecto-enzymes. This reagent, which proved to be successful in inactivating mouse macrophage NAD$^+$ glycohydrolase (Artman & Seeley, 1979), failed to affect NAD$^+$ glycohydrolase from calf splenocytes. Interestingly, a solubilized form of calf spleen NAD$^+$ glycohydrolase (Schuber *et al.*, 1980) was efficiently inactivated by diazosulphanilic acid, indicating that the enzyme is probably somewhat buried in the membrane (H. Muller, unpublished work). NAD$^+$ glycohydrolase from splenocytes could be inactivated by Woodward's reagent K (N-ethyl-5-phenylisoxazolium-3′-sulphonate), a non-permeant carboxy-group reagent (Pétra, 1971), and which we have previously shown to react with the active site of the enzyme (Schuber *et al.*, 1978). This inactivation was, however, accompanied by a substantial cell death.

Discussion

In the present work, we have attempted to provide evidence that NAD$^+$ glycohydrolase is an ecto-enzyme according to criteria previously discussed by De Pierre & Karnovsky (1973). For this cellular study we have adapted a fluorimetric assay of NAD$^+$ glycohydrolase, based on the observation that 1,N^6-etheno-NAD$^+$ is an excellent substrate of the calf spleen enzyme. This assay proved to be very reliable and sensitive. We have demonstrated that NAD$^+$ glycohydrolase associated with freshly isolated calf splenocytes is able to hydrolyse substrates added to the external medium. The substrates used, i.e. NAD$^+$ and 1,N^6-etheno-NAD$^+$, are not transported, and because of their charges do not readily diffuse through cellular membranes (Bishop *et al.*, 1959; Decker & Rupprecht, 1970; Liersch *et al.*, 1971; Plagemann & Wohlhueter, 1981). Moreover, we have also shown that intact cells were able to hydrolyse a polymer of NAD$^+$, a molecule whose structure and molecular weight make it impermeant to cells. The NAD$^+$ glycohydrolase activity, which is dependent on cell number, displays saturation kinetics, and the K_m value obtained for 1,N^6-etheno-NAD$^+$ is comparable with the values found for the solubilized (Schuber & Travo, 1976) and microsomal forms (Travo *et al.*, 1979) of the enzyme. These results indicate that the NAD$^+$-hydrolysing activity is expressed by an NAD$^+$ glycohydrolase whose active site is located on the outer surface of the splenocytes. In favour of such an interpretation is the finding that the rate of NAD$^+$ hydrolysis catalysed by the cells is similar to the rate of product accumulation in the extracellular medium. Finally, we showed that a Blue Dextran of M_r 40000, a potent non-permeant inhibitor of NAD$^+$ glycohydrolase, was able to block almost completely the activity of cellular NAD$^+$ glycohydrolase.

The NAD$^+$ glycohydrolase, which is a tightly membrane-bound enzyme (Schuber *et al.*, 1980), was associated with intact cells and its activity could not be accounted for by leaky or disrupted splenocytes. When the cells were permeabilized with digitonin, an increase in NAD$^+$ glycohydrolase activity could be obtained, i.e. 25% of total activity. This is a rather common observation, since several ecto-enzymes have been found distributed between the cell surface and internal pools, which can result for instance from the endocytic activity of cells. For example, about 20% of alkaline phosphodiesterase and 5′-nucleotidase, two ecto-enzymes of mouse macrophages, are not associated with the outer surface of the cellular membrane (Edelson & Cohn,

1976; Edelson & Erbs, 1978). The continuous recycling of plasma-membrane components, including enzymes (Stanley *et al.*, 1980; Widnell *et al.*, 1982), between the cell surface and e.g., endosomes, is now documented (Schneider *et al.*, 1979; Muller *et al.*, 1980; Tietze *et al.*, 1982; Widnell & Kitson, 1982) and NAD^+ glycohydrolase might behave similarly.

The evidence reported in the present paper demonstrates that NAD^+ glycohydrolase in calf spleen cells fulfils the criteria of an ecto-enzyme. Consistent with this conclusion is our observation that the hydrosoluble domain of NAD^+ glycohydrolase, which contains the active site (Schuber *et al.*, 1980), is a glycoprotein (I. Schenherr & F. Schuber, unpublished work).

The present work therefore proves unambiguously that the NAD^+-splitting activity by intact cells, which was observed previously (see the introduction), is due to an NAD^+ glycohydrolase activity associated with the cell surface. Moreover, at least in splenocytes, the catabolism of NAD^+ observed at physiological pH is mostly due to cleavage of the nicotinamide–ribose bond, the nucleotide pyrophosphatase activity becoming of importance only at higher pH values. The fact that NAD^+ glycohydrolase is an ecto-enzyme of certain mammalian cells, besides the well-documented case of erythrocytes, is certainly a far more general phenomenon than was hitherto recognized, e.g. NAD^+-splitting activity was reported in mouse macrophages (Artman & Seeley, 1979) and mentioned in fibroblasts (De Wolf *et al.*, 1981; Mandel *et al.*, 1981).

The occurrence of NAD^+ glycohydrolase as an ecto-enzyme is in contrast with the belief that this enzyme might be the main intracellular catabolic enzyme for $NAD(P)^+$ (see the introduction). As we have stressed (Muller & Schuber, 1980), the participation of the bulk cellular NAD^+ glycohydrolase in the turnover of intracellular NAD^+ can be largely ruled out. This finding has the merit of explaining the possible coexistence, in certain cells, of high activities of NAD^+ glycohydrolase and normal NAD^+ turnover (Bock *et al.*, 1968; Clark & Pinder, 1969; Bernofsky & Pankov, 1973).

The physiological role(s) of NAD^+ glycohydrolase remains to be elucidated. In this respect Artman & Seeley (1978) have observed that NAD^+ glycohydrolase is associated with mouse spleen and peritoneal macrophages. We have found a similar result with calf spleen macrophages, although it appears that the macrophages are not the unique source of NAD^+ glycohydrolase activity among the splenocytes. One possible role of NAD^+ glycohydrolase associated with, e.g., spleen macrophages could be the recycling of the nicotinamide moiety of $NAD(P)^+$ contained in the phagocytosed cells, or derived from cells killed by oxidative cytolysis by activated macrophages. This salvage pathway, which could be part of the general homeostatic function of the phagocytic cells (Silverstein *et al.*, 1977) bring us back to the original suggestion of Gholson (1966) about NAD^+ glycohydrolase, but with an enzyme which has its active site at the surface of the cells.

Finally, we emphasize that the availability of an easy and sensitive fluorescent assay for NAD^+ glycohydrolase, which can be performed at physiological pH in media which protect cellular integrity, should make this enzyme a useful new cell-surface marker.

The financing of this work by the Centre National de la Recherche Scientifique (LA 31 and ERA 487) and by the Délégation Générale de la Recherche Scientifique et Technique (AC Membranes) is gratefully acknowledged.

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