Localization of Oxidized Nicotinamide–Adenine Dinucleotide Glycohydrolase in the Mouse Liver Nuclear Envelope

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(Received 27 July 1978)

NAD⁺ glycohydrolase activity located in the nuclear envelope was maximally solubilized by treatment with 0.1–0.2% Triton X-100. The residual activity largely represents the chromatin-associated NAD⁺ glycohydrolase. Under these conditions the phospholipids were extensively solubilized (over 90%) while leaving the nuclei physically stable, although the nuclear membranes were removed, as shown by electron microscopy. After Triton X-100 treatment, deoxyribonuclease I did not significantly affect the residual NAD⁺ glycohydrolase activity, although the DNA was completely broken down. This enzyme activity can be released from the nuclear pellet by incubation with phospholipase C. For comparative studies, the glucose 6-phosphatase activity, known to be present in the nuclear envelope, was investigated. Treatment with 0.01% Triton X-100 released 10–20% of the phospholipids, but without solubilizing either glucose 6-phosphatase or NAD⁺ glycohydrolase. Higher Triton X-100 concentrations (0.1–1.0%) inhibited glucose 6-phosphatase, but not NAD⁺ glycohydrolase activity. NAD⁺ glycohydrolase is apparently present in a latent form in the nuclear envelope. Glucose 6-phosphatase, however, shows no such latency.

The role of NAD⁺ as a coenzyme in oxidoreduction systems is well established. Its participation as a metabolite in the regulation of cell proliferation is, however, less well characterized and open to speculation. Morton (1958) and Streffer & Scholz (1972) have shown that low intracellular concentrations of NAD⁺ are associated with high cell-proliferation rates in tumours and regenerating tissues.

NAD⁺ is hydrolysed by NAD⁺ glycohydrolase (EC 3.2.2.5) at the nicotinamide–ribose linkage with the formation of nicotinamide and ADP-ribose. Approx. 90% of the total cellular enzyme activity is found in the microsomal fraction; the remaining 10% is localized in the nucleus (Green & Mamaril, 1973).

Green & Dobrjansky (1972) and Green & Mamaril (1973) have shown that there are two NAD⁺ glycohydrolase activities associated with isolated Ehrlich-ascites-tumour-cell nuclei. One is associated with the chromatin, whereas the other is membrane-bound. The chromatin-associated NAD⁺ glycohydrolase activity has been variously described as being similar to, identical with and different from poly(ADP-ribose) polymerase [for reviews see Sugimura (1973) and Hilz & Stone (1976)]. This enzyme catalyses the formation of a polymer consisting of ADP-ribose

units (Sugimura *et al.*, 1976). The role of the polymer is unknown, although it may participate in the regulation of the function of chromatin (Shall *et al.*, 1977; Suhadolnik *et al.*, 1977). The NAD⁺ glycohydrolase activities associated with the nuclear membrane and microsomal fraction, which are apparently identical (Fukushima *et al.*, 1976), however, show no poly-(ADP-ribose) polymerase activity (Green & Dobrjansky, 1972).

Most of the reports on the isolation and solubilization of membrane-bound NAD⁺ glycohydrolase have involved organic solvents, enzymes (Schuber & Travo, 1976) and sonication followed by high-salt treatment (Green & Dobrjansky, 1972; Streffer & Beisel, 1974; Fukushima *et al.*, 1976). None has made use of the non-ionic detergent Triton X-100. We have thus examined the membrane-bound NAD⁺ glycohydrolase activity in mouse liver nuclei, using Triton X-100. A preliminary report of some of the studies in this paper was presented to the Austrian Biochemical Society (Streffer *et al.*, 1976).

Materials and Methods

Animals

The animals used were adult inbred male mice, 8–12 weeks old (strain: Radiologisches Institut,

Abbreviation used: DNAase, deoxyribonuclease.

Freiburg i. Br., W. Germany). They were fed on a standard diet and given acidified water (pH 3) *ad lib.* (Streffer & Scholz, 1972).

Preparation of subcellular fractions

Nuclei were isolated from mouse liver homogenates as described by Blobel & Potter (1966), without detergent treatment. Centrifugation was carried out in a Beckman L5-50 ultracentrifuge with a 60Ti rotor operating at 100000g for 60min at 3°C.

Counting of nuclei

After resuspension in STKM buffer [250mM-sucrose/50mM-Tris/HCl (pH 7.5)/25mM-KCl/5mM-MgCl₂], nuclei were counted with a haemocytometer.

Preparation of nuclear membranes and chromatin

The nuclear membranes and chromatin were isolated as described by Green & Dobrjansky (1972).

Reagents

[nicotinamide-¹⁴C]NAD⁺ (sp. radioactivity 59 mCi/mmol) was obtained from Amersham-Buchler, Braunschweig, W. Germany. Triton X-100 was from Serva, Heidelberg, W. Germany. All biochemicals were obtained either from Boehringer, Mannheim, or Sigma, Munich, W. Germany. All other reagents were of analytical grade from Merck, Darmstadt, W. Germany.

Enzyme assays

NAD⁺ glycohydrolase (EC 3.2.2.5) was assayed as described by Streffer & Beisel (1974), except that the reaction was stopped by chilling in ice at 4°C. To establish the linearity of the enzyme activity, each assay was incubated for 10 and 15 min and contained up to 10 µg of protein.

Glucose 6-phosphatase (EC 3.1.3.9) was assayed as described by Baginski *et al.* (1970). The reaction was terminated with 2.6 ml of 10% (w/v) trichloroacetic acid and the supernatant (1 ml) assayed for P_i by the method of Ames (1966).

Solubilization of NAD⁺ glycohydrolase by Triton X-100

Nuclei were incubated with 0.01–1.0% (v/v) Triton X-100 in STKM buffer, pH 7.5, at 4°C for 30 min. The samples were then centrifuged at 3000g for 10 min in a refrigerated Beckman J 21-B centrifuge. The supernatant was decanted and the pellet resuspended in STKM buffer. NAD⁺ glycohydrolase and glucose 6-phosphatase activities in both the supernatant and pellet fractions were assayed immediately.

Phospholipid determination

Nuclear pellets and supernatants of untreated and

Triton X-100-treated nuclei were extracted in chloroform/ethanol by the method of Hokin & Hokin (1958). This method assures that no non-lipid phosphorus is carried over (Ove *et al.*, 1971). Phosphorus was determined by the method of Ames (1966), modified as follows: 1 ml of chloroform extract containing phospholipids was evaporated to dryness and the residue digested and ashed completely with 0.5 ml of 70% (v/v) HClO₄ and not with Mg(NO₃)₂ as suggested in the original method.

Protein and DNA determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. DNA was measured by the method of Burton (1956) as described by Streffer (1974).

Radioactivity measurement

Radioactivity was measured in a B/F liquid-scintillation spectrometer, model 5000 (Berthold, Wildbad, W. Germany). The scintillation fluid contained 100 g of naphthalene and 5 g of Permablend 3 (Packard, Frankfurt, W. Germany) per litre of re-distilled dioxan. Values were converted into d.p.m. by automatic external standardization.

Electron microscopy

Nuclei were pelleted (3000g for 10 min), fixed in aq. 4–6% (v/v) glutaraldehyde (30 min) and post-fixed in 2% (w/v) OsO₄ (2 h). The pellets were then stained with 0.5% uranyl acetate, dehydrated in ascending alcohols and embedded in styrol. All preparations were examined in a Siemens M 200 electron microscope.

Results

Effects of Triton X-100 on NAD⁺ glycohydrolase activity

Incubation of the nuclear suspension with 0.01% Triton X-100 resulted in a slight but repeatedly found increase in NAD⁺ glycohydrolase activity of the resuspended nuclear pellet. The supernatant showed no increase in NAD⁺ glycohydrolase activity under these conditions. When the Triton X-100 concentration was increased to 0.2%, the NAD⁺ glycohydrolase activity in the pellet was decreased to about 40–50% of the control value (Figs. 1 and 2). At the same time the activity in the supernatant reached a maximum after an incubation time of 30 min. No additional significant amounts of NAD⁺ glycohydrolase were solubilized, nor was the activity in the pellet further affected, with Triton X-100 concentrations up to 1.0%. When the sum of the activities in the 0.2% Triton X-100-treated pellet and supernatant is compared with the sum of the control

values, a 2-fold increase in NAD⁺ glycohydrolase activity is found.

Effect of Triton X-100 on protein determination

Under the present experimental conditions, Triton X-100 up to 0.5% final concentration did not interfere with the Lowry *et al.* (1951) procedure for protein determination. This was confirmed by separate control experiments. At higher concentrations Triton X-100 produced turbid solutions and insoluble complexes, making absorbance measurements impossible.

Effect of Triton X-100 on NAD⁺ glycohydrolase activity in the nuclear membrane and chromatin preparations

The effect of treatment of the nuclear suspension

Fig. 1. NAD⁺ glycohydrolase activity in mouse liver nuclei and supernatant after Triton X-100 treatment

Liver nuclei were incubated with Triton X-100 for 30 min at 4°C, centrifuged and resuspended in STKM buffer, pH 7.5. NAD⁺ glycohydrolase activity in nuclei (●) and supernatant (○), expressed as 10⁻⁶ × d.p.m./h per g of tissue, was assayed as described in the Materials and Methods section.

with 0.1% Triton X-100 and its subsequent fractionation into the membrane and chromatin preparation as described by Green & Dobrjansky (1972) is shown in Table 1.

Although only about 18% loss of the specific NAD⁺ glycohydrolase activity is found on sonication with a Branson Sonifier (Plainview, NY, U.S.A.; 70 W, 30 s) of both the Triton X-100-treated and untreated nuclear suspensions, almost 50% of the specific activity is lost after addition of NaCl in both cases (Table 1). The remaining NAD⁺ glycohydrolase activities in both the soluble chromatin and nuclear membrane fractions per g of tissue are completely recoverable from the NaCl-treated sonicated preparations. The nuclear membrane fraction constitutes about 80% of the total NAD⁺ glycohydrolase activity in both the untreated and Triton X-100-treated sonicated preparations in 2M-NaCl. The Triton X-100-treated nuclear membrane fraction contains about 25% of the specific NAD⁺ glycohydrolase activity compared with the untreated nuclear membrane fraction, the remainder having been solubilized. The specific NAD⁺ glycohydrolase activities in the soluble and precipitated chromatin fractions are both essentially unaffected after Triton X-100 treatment.

Effect of Triton X-100 on glucose 6-phosphatase and phospholipids

Fig. 2 shows the effect of 0.01–1.0% Triton X-100 on both the glucose 6-phosphatase activity and phospholipids. The phospholipids were removed to leave about 80% of their control value at 0.01% Triton X-100, whereas the glucose 6-phosphatase activity was found to be either unaffected or slightly stimulated in a manner similar to NAD⁺ glycohydrolase. At 0.05% Triton X-100, over 90% of the phospholipids were solubilized from the nuclear pellet and quantitatively recovered in the supernatant, but the

Table 1. NAD⁺ glycohydrolase activity in nuclear fractions from mouse liver

Nuclei were prepared by the method of Blobel & Potter (1966), incubated with 0.1% Triton X-100 for 30 min at 4°C and fractionated into nuclear membranes and chromatin as described by Green & Dobrjansky (1972). NAD⁺ glycohydrolase activity was assayed as described in the Materials and Methods section. Values in parentheses are expressed as percentages of that in the nuclear pellet.

Fraction	NAD ⁺ glycohydrolase activity			
	-Triton X-100		+Triton X-100	
	(10 ⁻⁶ × d.p.m./h per mg of protein)	(10 ⁻⁶ × d.p.m./h per g of liver)	(10 ⁻⁶ × d.p.m./h per mg of protein)	(10 ⁻⁶ × d.p.m./h per g of liver)
Nuclear pellet	38.6 (100)	29.3 (100)	10.5 (100)	7.9 (100)
Nuclear pellet+sonication	31.4 (81)	22.9 (78)	8.7 (83)	7.0 (88)
Sonicated product+2M-NaCl	20.3 (53)	15.8 (54)	6.1 (58)	4.6 (58)
Nuclear membrane	73.9 (191)	13.3 (45)	20.6 (196)	3.5 (44)
Soluble chromatin	2.7 (7)	1.7 (6)	1.6 (15)	1.0 (13)
Precipitated chromatin	3.1 (8)	0.4 (1)	2.5 (24)	0.4 (5)

residual 10% could not be removed by further treatment with Triton X-100 up to 1.0%. Glucose 6-phosphatase activity at 0.05% Triton X-100 was decreased to about 50% in the nuclear pellet; the remainder could be completely recovered in the supernatant. On increasing the Triton X-100 concentration to 0.1%, over 70% of the glucose 6-phosphatase was removed, but only about 30% of the activity could be found in the supernatant. At 0.2–1.0% Triton X-100, a constant 10% of the activity remained

in the pellets, whereas the enzyme activity in the 1.0% Triton X-100 supernatant was totally inactivated.

Electron microscopy

Light-microscopy after treatment with Triton X-100 revealed no distinct nuclear morphological changes. Electron microscopy, however, showed the outer nuclear membrane to be severely damaged and to be intact at only a few sites on the nuclei after treatment with 0.01% Triton X-100. At 0.1% Triton X-100, the nuclear envelope was completely detached from the nuclei. In spite of lacking a ‘visible’ membranous system, the nuclei still retained their characteristic ‘round’ shape (Plate 1).

Action of phospholipase on NAD⁺ glycohydrolase activity

Phospholipases A (*Crotalus terrificus terrificus*, Boehringer no. 108499), C (*Bacillus cereus*, Boehringer no. 108502) and D (cabbage, Boehringer no. 108529) were used in an attempt to solubilize NAD⁺ glycohydrolase in a manner similar to that of Triton X-100. The enzyme activities used were so chosen so as not to destroy the nuclei. Only phospholipase C at concentrations up to 80 units (μmol/min)/ml acting on 30×10⁶ nuclei solubilized NAD⁺ glycohydrolase without destroying the gross nuclear morphology on incubation at 37°C for 30min (Table 2). Treatment with phospholipases A and D (2 units/ml and 0.06 unit/ml respectively; 30×10⁶ nuclei), however, did not produce any significant solubilization under similar conditions (results not shown). Phospholipase C (16 and 80 units/ml) respectively solubilized 16 and 45% (5.2×10⁶ and 14.7×10⁶d.p.m.) of the initial specific NAD⁺

Fig. 2. Effect of Triton X-100 on NAD⁺ glycohydrolase and glucose 6-phosphatase activities and phospholipid content in mouse liver nuclei

Liver nuclei were incubated with Triton X-100 for 30min at 4°C, centrifuged and resuspended in STKM buffer, pH7.5. NAD⁺ glycohydrolase (●), glucose 6-phosphatase (○) and phospholipid content (□) were assayed as described in the Materials and Methods section. Data are given as percentages of the control values.

Table 2. NAD⁺ glycohydrolase activity of untreated and 0.1%-Triton X-100-treated mouse liver nuclei after incubation with phospholipase C

Experimental details are as described in the text. Nuclear pellet 2 and supernatant 2 were obtained by centrifugation after treatment of nuclear pellet 1 with phospholipase C.

			NAD ⁺ glycohydrolase (10 ⁻⁶ × d.p.m./h per mg of protein)			Phospholipid content (μg P ₁ /g of liver)	
Phospholipase C treatment ...			Control	16 units	80 units	Control	16 units
Without Triton X-100	Pellet 1	Pellet 2		29.7	8.1		0.54
		Supernatant 2	32.9	5.2	14.7	4.33	Trace
	Supernatant 1		8.8	—	—	0.26	0
With Triton X-100 (0.1%)	Pellet 1	Pellet 2		18.0	4.1		0.52
		Supernatant 2	14.1	2.4	7.4	0.87	0.41
	Supernatant 1		70.4	55.4	23.9	4.19	0.37

EXPLANATION OF PLATE I

Electron micrographs of intact (a) and 0.1%-Triton X-100-treated mouse liver nuclei (b)
Magnifications: a, $\times 12500$; b, $\times 7300$. Nuclei were incubated with Triton X-100 at 4°C for 30 min.

glycohydrolase activity (32.9×10^6 d.p.m.), although the recovery of total NAD⁺ glycohydrolase activity at the latter concentration was only 70%. Since 0.1–0.2% Triton X-100 produced maximal solubilization of NAD⁺ glycohydrolase while at the same time leaving a pellet still retaining about 50% of the initial NAD⁺ glycohydrolase activity, it was decided to see whether phospholipase C could lead to further release of NAD⁺ glycohydrolase. Phospholipase C released 17 and 52% (2.4×10^6 and 7.4×10^6 d.p.m.) of the activity from the treated pellet (14.1×10^6 d.p.m.) under the above-mentioned conditions. The NAD⁺ glycohydrolase activity in the Triton X-100 supernatant was decreased by over 60% only at the highest phospholipase C concentration. Treatment with 16 units of phospholipase C/ml resulted in the hydrolysis of 90% of the phospholipids present in the control nuclear pellet. No phospholipids could be detected in the supernatant. Incubation of the Triton X-100-treated nuclear pellet, containing 20% of the total phospholipids ($0.87 \mu\text{g}$ of phosphate/g), with phospholipase C resulted in the solubilization of a further 47% of these remaining phospholipids. The rest were retained in the pellet (Table 2). No hydrolysis of the phospholipids occurred under these conditions. The phospholipids in the original Triton X-100 supernatant, on the other hand, were degraded by 90%.

Effect of DNAase I on NAD⁺ glycohydrolase activity

Nuclear pellets, before and after 0.1% Triton X-100 treatment, were incubated with pancreatic DNAase I (Boehringer no. 104159), pH 7.5 in STKM buffer, at 37°C for 30 min. DNAase I (5000 units/ml, with 30×10^6 nuclei) completely hydrolysed the DNA in both the control and Triton X-100-treated pellets, whereas at a concentration of 1000 units/ml (30×10^6 nuclei) the pellets still possessed up to 20% of their DNA content. The NAD⁺ glycohydrolase activities in both the untreated and Triton X-100-treated pellets were not significantly affected by both the DNAase concentrations used. In all cases about 20% of the initial NAD⁺ glycohydrolase was solubilized, but the activity was completely recovered.

The NAD⁺ glycohydrolase activity in the supernatants, too, was found to be insensitive to DNAase. Both the untreated and Triton X-100-treated nuclei appeared morphologically intact on light- or phase-contrast microscopy, although diminished in size and volume.

Discussion

From the studies by Green & Dobrjansky (1972) on Ehrlich-ascites-tumour cells, to Streffer and co-workers (Streffer & Beisel, 1974; Konings *et al.*,

1975; Streffer & Schmiebusch, 1975) on mouse spleen and liver as well as Ueda *et al.* (1975) on rat liver, it appears that the NAD⁺ glycohydrolase activities associated with chromatin and nuclear membrane are separate enzymic entities. Whereas according to Green & Dobrjansky (1972) the chromatin-associated NAD⁺ glycohydrolase activity has been postulated to possess poly(ADP-ribose) polymerase activity and may thus play a direct part in cellular proliferation, the role of the nuclear-membrane-bound activity is unknown.

The isolation and localization of the nuclear-membrane-bound NAD⁺ glycohydrolase therefore appears to be a prerequisite for further advances in our understanding of the physiological function of this particular enzyme. Previously used methods for isolating this enzyme have utilized sonication and salt treatment; this, however, has the disadvantage that the nuclei are totally destroyed. This makes morphological studies on the localization of the enzyme activity extremely difficult and furthermore results in large losses of NAD⁺ glycohydrolase activity after salt treatment. We have overcome this problem by using the detergent Triton X-100, which leaves the nuclei membrane-denuded but 'intact' with respect to shape, thus facilitating the correlation of morphological with biochemical data.

In the present study we were able to solubilize maximally the nuclear-membrane-bound NAD⁺ glycohydrolase activity with 0.1–0.2% Triton X-100. The resulting nuclear pellet contains the chromatin-associated NAD⁺ glycohydrolase activity, since the nuclear envelope is extensively degraded, as demonstrated by the solubilization of phospholipids, considered to be a good marker for nuclear membranes (Aaronson & Blobel, 1974; Monneron *et al.*, 1972), and electron microscopy. The soluble and precipitated chromatin NAD⁺ glycohydrolase activities as well as the DNA contents are unaffected. Under these conditions, phospholipids were extensively solubilized and yet these apparently membraneless nuclei remained physically stable, which agrees with the previous findings (Aaronson & Blobel, 1974; Coetzee *et al.*, 1975; Keller & Riley, 1976) that the bulk of the phospholipids are not required for the maintenance of the nuclear structure.

Since Triton X-100 apparently does not selectively remove the outer from the inner membrane (Franke, 1974; Franke & Scheer, 1974), it is extremely difficult to say exactly where the NAD⁺ glycohydrolase activity is located in the nuclear envelope. Electron- and light-microscope studies showed the nuclei to be free from cytoplasmic contamination. Kartenbeck *et al.* (1973) and Sikström *et al.* (1976) have suggested that glucose 6-phosphatase, a well-known microsomal marker enzyme, may be inherent to the nuclear membrane system. This was confirmed in the present studies. Therefore glucose 6-phosphatase was assayed

as a further measure of membrane solubilization by Triton X-100, in addition to the solubilization of phospholipids. The solubilization of phospholipids always preceded the removal of both enzymes on increasing Triton X-100 concentrations, although glucose 6-phosphatase was maximally solubilized at a lower concentration than NAD⁺ glycohydrolase. The loss of glucose 6-phosphatase activity appears to be due to denaturation or conformational changes of the enzyme with irreversible inhibition (Garland & Cori, 1972; Helenius & Simons, 1975) and independent of the phospholipid content.

NAD⁺ glycohydrolase appears to be partially present in a latent form in the nuclear membrane. Shushevych *et al.* (1975) have also reported an overall stimulatory effect of up to 0.1% Triton X-100 on NAD⁺ glycohydrolase activity in rat liver nuclei, without separating the nuclear suspension into a pellet and supernatant fraction. The chromatin-associated NAD⁺ glycohydrolase activity of Ehrlich ascites cells was completely inactivated by DNAase treatment (Green & Dobrjansky, 1972), whereas the enzyme from rat liver was insensitive (Ueda *et al.*, 1975). The corresponding membrane-bound NAD⁺ glycohydrolase activities were unaffected by DNAase treatment (Green & Dobrjansky, 1972; Fukushima *et al.*, 1976). Shushevych *et al.* (1975) found that DNAase treatment of intact rat liver nuclei led to a 50% loss of NAD⁺ glycohydrolase activity. In contrast, we have found that treatment with DNAase I had no significant effect on the NAD⁺ glycohydrolase activity in either the pellets or supernatants, in spite of the complete hydrolysis of DNA.

Our data suggest that the chromatin-associated NAD⁺ glycohydrolase activity is not dependent on the nuclear DNA. Ueda *et al.* (1975) have reported on the existence of a discrete NAD⁺ glycohydrolase in addition to poly(ADP-ribose) polymerase, possibly associated with the nucleolus. We have, however, only found a minimal amount of NAD⁺ glycohydrolase activity associated with isolated nucleoli.

The action of phospholipase C on intact nuclei with respect to solubilization of phospholipids closely resembles that of Triton X-100. The chromatin-associated NAD⁺ glycohydrolase, although not further affected by increasing Triton X-100 concentrations, could be substantially solubilized by treatment with phospholipase C.

Whereas the phospholipids from intact nuclei are hydrolysed, those of the Triton X-100-treated pellet are solubilized. It is noteworthy that both the untreated and Triton X-100-treated pellets contain exactly the same amount of residual phospholipid after phospholipase C treatment. This suggests that these phospholipids are probably intranuclear and may consist of phospholipids not accessible to phospholipase C.

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