Topography, purification and characterization of thyroidal NAD⁺ glycohydrolase

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Subcellular fractionation of bovine thyroid tissue by differential pelleting and isopycnic gradient centrifugation in a zonal rotor indicated that NAD⁺ glycohydrolase is predominantly located and rather uniformly distributed in the plasma membrane. Comparison of NAD⁺ glycohydrolase activities of intact thyroid tissue slices, functional rat thyroid cells in culture (FRT_L) and their respective homogenates indicated that most if not all of the enzyme (catalytic site) is accessible to extracellular NAD⁺. The reaction product nicotinamide was predominantly recovered from the extracellular medium. The diazonium salt of sulphanilic acid, not penetrating into intact cells, was able to decrease the activity of intact thyroid tissue slices to the same extent as in the homogenate. Under the same conditions this reagent almost completely abolished NAD⁺ glycohydrolase activity associated with intact thyroid cells in culture. The triazine dye Cibacron Blue F3GA and its high-*M_r* derivative Blue Dextran respectively completely eliminated or caused a severe depression in the NAD⁺ glycohydrolase activity of FRT_L cells. The enzyme could be readily solubilized from bovine thyroid membranes by detergent extraction, and was further purified by gel filtration and affinity chromatography on Blue Sepharose CL-6B. The overall procedure resulted in a 1940-fold purification (specific activity 77.6 μmol of nicotinamide released/h per mg). The purified enzyme displays a *K_m* of 0.40 mM for β-NAD⁺, a broad pH optimum around pH 7.2 (0.1 M-potassium phosphate buffer) and an apparent *M_r* of 120 000. Nicotinamide is an inhibitor (*K_i* 1.9 mM) of the non-competitive type. The second reaction product ADP-ribose acts as a competitive inhibitor (*K_i* 2.7 mM). The purified enzyme splits β-NAD⁺, β-NADP⁺, β-NADH and α-NAD⁺ at rates in the relative proportions 1:0.75:<0.02:<0.02 and exhibits transglycosidase (pyridine-base exchange) activity. Anionic phospholipids such as phosphatidylinositol and phosphatidylserine inhibit the partially purified enzyme. A stimulating effect was observed upon the addition of histones.

Apart from its major biochemical function as an electron carrier in various biological oxidation-reduction systems, NAD⁺ serves as a substrate for several NAD⁺-transforming activities such as NAD⁺ glycohydrolase (NADase, EC 3.2.2.6), ADP-ribosyltransferase and poly(ADP-ribose) polymerase (EC 2.4.99.-). The observation that several bacterial toxins such as diphtheria toxin, cholera toxin and heat-labile enterotoxin of *Escher-*

ichia coli exert their effects by catalysing the transfer of ADP-ribose from NAD⁺ to specific intracellular acceptor proteins (Moss & Vaughan, 1979) has promoted a new interest in these NAD⁺-transforming enzymes. Evidence has accumulated suggesting that the glycoprotein hormone thyrotropin (TSH) is structurally and functionally related to cholera toxin (Kohn *et al.*, 1981). However, clear-cut discrepancies exist between the mode of action of these agents (Van Sande *et al.*, 1977, 1979). In contrast with cholera toxin, neither TSH nor its α-subunit has intrinsic ADP-ribosyltransferase and

Abbreviations used: NADase, NAD⁺ glycohydrolase; TSH, thyrotropin (thyroid-stimulating hormone).

NADase activities (Moss *et al.*, 1978). However, in bovine thyroid membranes the presence of both ADP-ribosyltransferase and NADase activity could be demonstrated (De Wolf *et al.*, 1981*b*). In subsequent studies the potential role of these enzymes in the mechanism of TSH message transmission (Vitti *et al.*, 1982; De Wolf *et al.*, 1983; Rebois *et al.*, 1983) and agonist (TSH)-induced refractoriness (Filetti & Rapoport, 1981; Rapoport *et al.*, 1982) was investigated. In the present investigation we have studied the subcellular localization and the topographical distribution of thyroidal NADase in order to define its precise site of action. We also report on the development of a procedure for solubilization and purification of this enzyme and the characterization of some of its properties.

Parts of this work have been previously published in abstract form (De Wolf *et al.*, 1981*c*, 1982*a,b*).

Materials and methods

Chemicals

Cholera toxin was purchased from Schwarz-Mann Laboratories. L-Arginine methyl ester, β -NAD⁺, phenyl-Sepharose CL-4B, β -NAD⁺-agarose, insulin, cortisol (hydrocortisone), transferrin, NADP⁺, NADH, cyclic AMP, ATP and ADP were from Sigma Chemical Co., *Lens culinaris* lectin-Sepharose 4B, 5'-AMP-Sepharose 4B, Blue Sepharose CL-6B, Sephadex G-200 (particle size 40–120 μ m) were from Pharmacia, Dowex AG-1-X2 (100–200 mesh, Cl⁻ form) and Bio-Gel P-200 (200–400 mesh) were from Bio-Rad Laboratories and sulphanilic acid was from Merck. The diazonium salt of sulphanilic acid was prepared as described by Berg (1969). Crude TSH was obtained from Armour Laboratories (Chicago, IL, U.S.A.). Purified TSH was generously given by Dr. L. D. Kohn (National Institutes of Health, Bethesda, MD, U.S.A.). In a McKenzie mouse bioassay, its activity amounted to 23 ± 2 i.u./mg of protein. Glycyl-L-histidyl-L-lysine acetate and somatostatin were from Calbiochem, and Procion Blue HB (Cibacron Blue F3GA), *NN*-diethylnicotinamide and acetylpyridine were from Janssen Chimica (Beerse, Belgium). Acridine Orange and ethidium bromide were obtained from Aldrich Europe. [*carboxyl*-¹⁴C]NAD⁺ (approx. 50 mCi/mmol) and [*carboxyl*-¹⁴C]nicotinamide (approx. 50 mCi/mmol) were from Amersham International

Cell culture

Functioning (concentration of I⁻ and production of thyroglobulin) rat thyroid cells (FRT_L) (Vitti *et al.*, 1982) were grown in Coon's modified Hanks F-12 medium (Ambesi-Impimbatto *et al.*, 1980)

supplemented with 0.5% foetal calf serum and a hormone mixture consisting of insulin (10 μ g/ml), cortisol (10 nM), transferrin (5 μ g/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), somatostatin (10 ng/ml) and TSH (10 m-i.u./ml). Cells were grown in a CO₂/air (1:19) atmosphere at 37°C. Normal skin fibroblasts (CRL 1141) and mouse myeloma cells (P3-X63-Ag8) were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% foetal-calf serum.

Cell fractionation

Subcellular fractionation of fresh bovine thyroid glands by differential pelleting was performed as described previously (Hilderson *et al.*, 1975). Isopycnic gradient centrifugation of a combined M + L fraction was performed in an HS zonal rotor as previously reported (De Wolf *et al.*, 1978*a*). Rat liver microsomal fraction was prepared as described by Sedwick & Hübscher (1965).

Enzyme assays

NADase activity was measured by following the formation of [*carboxyl*-¹⁴C]nicotinamide from [*carboxyl*-¹⁴C]NAD⁺ as previously described (De Wolf *et al.*, 1981*b*). All assays were performed in duplicate. When unlabelled substrates were used the enzyme activity was determined by measuring the cyanide addition product by the method of Kaplan (1955). The hydrolysis of NADH was determined by measuring residual NADH with lactate dehydrogenase in the presence of pyruvate (Skidmore & Trams, 1970). Transglycosidase activity was assayed by measuring the incorporation of externally added [¹⁴C]nicotinamide into NAD⁺. The reaction mixture consisted of 100 mM-Tris/HCl buffer, pH 7.5, 0.1 mM-NAD⁺, 5 mM-[*carboxyl*-¹⁴C]nicotinamide (53 mCi/mol) and enzyme in a total volume of 0.2 ml. Reactions were terminated by dilution with 1 ml of ice-cold 20 mM-Tris/acetate buffer, pH 7.0, the incubation mixture was applied to Dowex AG-1-X2 columns and, after elution of [*carboxyl*-¹⁴C]nicotinamide with 10 ml of 20 mM-Tris/acetate buffer, pH 7.0, [*carboxyl*-¹⁴C]NAD⁺ was eluted with 5 ml of 0.1 M-HCl.

ADP-ribosyltransferase activity was assayed by measuring the formation of [*carboxyl*-¹⁴C]nicotinamide from [*carboxyl*-¹⁴C]NAD⁺ in the presence of 60 mM-L-arginine methyl ester (De Wolf *et al.*, 1981*b*) or by using *p*-nitrobenzylidene amino guanidine as substrate (Soman *et al.*, 1983).

Alkaline phenylphosphatase activity was determined by the method of Kind & King (1954) at pH 10. 5'-Nucleotidase activity was assayed by using the procedure of Morré (1973). Guaiacol peroxidase activity was measured as previously described (De Wolf *et al.*, 1978*b*). Adenylate cyclase activity was assayed as described elsewhere

(De Wolf *et al.*, 1981a). Hexosaminidase was measured by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl α -D-2-acetamido-2-deoxy- β -D-glucopyranoside (Li & Li, 1970). Lactate dehydrogenase was determined as described by Schwert & Winner (1963). Several other markers and marker enzymes were assayed as described previously (De Wolf *et al.*, 1978a). A total histone fraction was prepared from bovine thyroid nuclei (Widnell & Tata, 1964) by the procedure of Bonner *et al.* (1968). Proteins were determined by the method of Lowry *et al.* (1951) or fluorimetrically (Sims & Carnegie, 1975), with bovine serum albumin as a standard.

Purification of NADase

Step 1: preparation of cell homogenate. Anatomically prepared and iso-osmotically washed thyroid tissue blocks were homogenized in 4 vol. of 5 mM-Tris/HCl buffer, pH 7.4, containing 0.25 M-sucrose in a Waring Blendor (high speed, 30 s at 40°C). Portions (25 ml) of the resulting suspension were homogenized in a Potter-Elvehjem homogenizer (Teflon pestle; 3000 rev./min, six strokes).

Step 2: preparation of a combined mitochondrial and light-mitochondrial (M+L) fraction. To remove blood cells, connective tissue, cell debris and nuclei, the homogenate was centrifuged at 1000g for 3 min in a MSE Mistral 6L centrifuge. The ensuing supernatant was aspirated and centrifuged at 73 300g (6 × 100 ml angle rotor, MSE 18 centrifuge) for 10 min, resulting in an M+L pellet.

Step 3: osmotic shock. The M+L pellet was suspended in 200 ml of 1 mM-Tris/HCl buffer, pH 7.4, in a Potter-Elvehjem homogenizer (3000 rev./min, three strokes). The suspension was left on ice for 1 h and subsequently centrifuged at 144 700g (MSE 65 centrifuge) for 1 h.

Step 4: solubilization. The hypo-osmotically washed M+L sediment was suspended in 25 ml of 10 mM-Tris/HCl buffer, pH 7.4, containing 2% (w/v) Triton X-100 and 1% (w/v) sodium deoxycholate and rehomogenized in a Potter-Elvehjem homogenizer (3000 rev./min, five strokes). The suspension was subjected to ultrasonic disintegration in a Braun-Sonic 300 (setting 35, 1 min). During sonication the suspension was chilled on crushed ice. The sonicated preparation was left at room temperature for 1 h and subsequently centrifuged at 144 700g (MSE ultracentrifuge) for 1 h. The resulting sediment was discarded. The supernatant was filtered through Whatman no. 1 filter paper.

Step 5: gel chromatography. Gel chromatography was performed on a Sephadex G-200 (particle size 40–120 μ m) column (2.5 cm × 180 cm). The column was equilibrated with 10 mM-Tris/HCl buffer, pH 7.4, containing 1% Triton X-100 and 0.2%

sodium deoxycholate and eluted with the same solvent at 4°C. Fractions (5 ml) were collected at a flow rate of 14 ml/h.

Step 6: acetone precipitation. The Sephadex G-200 fractions containing the NADase activity were pooled and subjected to acetone precipitation. To this end 5 vol. of cold acetone (< -20°C) was added and the mixture was immediately centrifuged at 2000g_{max}. (Mistral 6L, temperature control at -20°C) for 10 min. The sediment was suspended in 5 ml of 10 mM-Tris/HCl buffer, pH 7.4, and stirred for 3 h at 4°C.

Step 7: affinity chromatography on Blue Sepharose CL-6B column (2.0 cm × 6.0 cm). The column was equilibrated with 10 mM-Tris/HCl buffer, pH 7.4. After application of the sample the column was eluted with 100 ml of Tris/HCl buffer, pH 7.4, and subsequently with a linear gradient of NaCl (200 ml of 10 mM-Tris/HCl buffer, pH 7.4, containing 1 M-NaCl in the reservoir; 200 ml of 10 mM-Tris/HCl buffer, pH 7.4, in the mixing chamber). Fractions (5 ml) were collected at a flow rate of 50 ml/h.

Results

Subcellular localization

Upon fractionation of a bovine thyroid homogenate by differential pelleting, NADase activity was mainly recovered in the M (mitochondrial) and L (light-mitochondrial) fractions. As indicated in Fig. 1, the highest relative specific activity was found in the L fraction (recovery $95 \pm 7\%$). Only plasma-membrane markers such as 5'-nucleotidase, alkaline phosphatase, adenylate cyclase, cholesterol and lipid-bound sialic acid also have their highest relative specific activity in this fraction (Hilderson *et al.*, 1975). A similar distribution pattern would suggest that the thyroidal NADase is predominantly located in the plasma membrane. More precise information about the subcellular localization was obtained by subjecting an M+L fraction to isopycnic gradient centrifugation in an HS zonal rotor. From Fig. 2 it is seen that NADase is distributed throughout the whole gradient, with peaks at densities 1.071 and 1.150. The peak at the lower density coincides with 5'-nucleotidase. The peak at higher density on the other hand coincides with alkaline phosphatase. The profile of NADase fails to follow the distribution pattern obtained for endoplasmic-reticulum markers such as guaiacol peroxidase and glucose-6-phosphatase (not indicated in Fig. 2). Also, lysosomal markers such as hexosaminidase and the mitochondrial marker cytochrome *c* oxidase all display a different distribution. Although the zonal profiles overlap each other very much, these results provide further evidence for a localization in the

Fig. 1. Distribution patterns of NADase and some markers and marker enzymes after differential centrifugation of thyroid homogenate

Fractions from left to right: N, nuclear fraction; M, mitochondrial fraction; L, light-mitochondrial fraction; P, microsomal fraction; S, soluble fraction. Ordinate: relative specific activity for enzymes; relative specific amount (% distribution of marker compound divided by % distribution of protein) for other markers. Abscissa: % of total protein recovered in each fraction.

plasma membranes of the thyroidal NADase. Furthermore, the enzyme activity appears to be uniformly distributed over the apical (luminal) and basolateral (antiluminal) sites of the plasma membrane of the epithelial thyroid cell.

Topography of NADase

Before study of the topology of NADase in the plasma membrane of thyroid epithelial cells several experimental conditions were established.

Viability of the thyroid cells in monolayer culture was monitored by fluorescence microscopy, with ethidium bromide and Acridine Orange as counterstain (Lee *et al.*, 1975). Before each assay 99% of the cells showed a green fluorescence. This value was not significantly altered (>95%) after enzyme assay or cell treatment. Lactate dehydrogenase activity was measured to monitor cell breakage and used as an internal indicator of reagent penetration. In thyroid, as in most other

Fig. 2. Zonal centrifugation of a 73300g pellet from an NaHCO₃ homogenate in an HS zonal rotor: distribution profiles of NADase and some marker enzymes

Experimental conditions were as follows. A 100g portion of bovine thyroid tissue was minced in a meat mill and made up to 250ml with 60mM-NaHCO₃, pH 7.4 (containing 3mM-EGTA). The tissue was further homogenized in a Waring Blendor (30s, maximum speed). The homogenate was centrifuged in an MSE Mistral 6L centrifuge (1000rev./min for 10min) and the resulting pellet discarded. Subsequently the supernatant was centrifuged at 73300g_{max} in an MSE 25 high-speed centrifuge (8 × 50ml angle rotor) for 15min. The ensuing sediment was resuspended in 12ml of 0.25M-sucrose (in 5mM-imidazole/HCl buffer, pH 7.4, containing 1mM-NaHCO₃ and 5mM EGTA) and applied on top of a linear sucrose gradient [15–50% (w/w), linear with respect to volume; additions as in the sample preparation; prepared in an MSE gradient former] in a HS zonal rotor, and the gradient was centrifuged at 10400g_{max} for 18h 30min. At the end of the centrifugation period the zonal rotor was unloaded by displacement with 55% (w/w) sucrose solution. As a routine 38 fractions (20ml) were collected. The fractions were analysed for enzymic activities and chemical composition. (a) ●, Slope of gradient; ■, proteins. (b) ○, Alkaline phosphatase; ●, NADase; △, 5'-nucleotidase. (c) ▲, β-D-N-Acetylglucosaminidase; □, guaiacol peroxidase; ▽, cytochrome c oxidase.

tissues, this enzyme is a soluble cytosolic protein. Upon differential pelleting of bovine thyroid tissue about 95% of the lactate dehydrogenase activity was recovered in the supernatant fraction. This fraction also displayed the highest relative specific activity (1.1) for this enzyme. Breakage of the cells would release this enzyme into the medium. As shown in respectively Tables 1 and 2 11% of the activity was released into the medium when thyroid slices were used and less than 5% with thyroid cells in culture.

It has been shown previously that NAD⁺ is not able to penetrate through the cell membrane unless it is degraded to at least the ribonucleoside level (Deguchi *et al.*, 1968; Kakehi *et al.*, 1972). With thyroid cells in culture, after incubation with [carbonyl-¹⁴C]NAD⁺ not more than 1% of the isotopic label had accumulated inside the cell after a 30min incubation period.

Comparison of NADase activity of intact thyroid-tissue slices with that of their homogenates revealed no significant difference (Table 1). Because of the nature of the assay, the reaction product (nicotinamide) is recovered from the extracellular medium when intact tissue is used. No extensive cell breakage occurred during the course of these experiments, as indicated by the lactate dehydrogenase activity. Homogenization of thyroid slices, however, produced a significant increase (+25%) in 5'-nucleotidase activity. These results suggest that most of the activity of NADase and about 75% of 5'-nucleotidase are the result of catalysis occurring at the exterior surface of the thyroid cell.

Further evidence was obtained by treatment of thyroid slices with the diazonium salt of sulphanilic acid. The diazonium salt of sulphanilic acid, reagent (Berg, 1969; Bender *et al.*, 1971), is highly reactive and forms covalent bonds with several functional groups in proteins (Vallee & Riordan, 1969). It has been useful in the study of the topology of plasma-membrane enzymes (De Pierre & Karnovsky, 1974). Treatment of bovine thyroid slices (0.5g of tissue) with the diazonium salt of sulphanilic acid (5mM) in Krebs-Ringer/Tris (KRT) buffer was performed at 37°C for 16min (this buffer contained 120mM-NaCl, 1.4mM-CaCl₂, 5.2mM-KCl, 1.4mM-MgSO₄ and 5mM-Tris/acetate, pH 7.2). After incubation the supernatant was aspirated and slices were washed ten times with KRT buffer. After treatment slices were assayed for NADase, 5'-nucleotidase and lactate dehydrogenase activity as described in the Materials and methods section. As indicated in Table 1, NADase and 5'-nucleotidase activities of intact thyroid slices were susceptible to inhibition by the diazonium salt of sulphanilic acid. Lactate dehydrogenase activity, however, was hardly

Table 1. *Effect of treatment with the diazonium salt of sulphanilic acid on NADase, 5'-nucleotidase and lactate dehydrogenase activities of intact and disrupted bovine thyroid slices*

Bovine thyroid slices were incubated at 37°C with the diazonium salt of sulphanilic acid at a concentration of 5.0 mM for 16 min. After treatment, slices were washed ten times with 20 mM-Hepes/NaOH buffer, pH 7.4, containing 0.9% NaCl. Specific activities in disrupted untreated tissue (= 100%): NADase, 0.04 µmol/h per mg of protein; 5'-nucleotidase, 0.61 µmol/h per mg of protein, lactate dehydrogenase, 0.28 µmol/h per mg of protein.

Enzyme	Specific activity (% of that in disrupted untreated tissue)				
	Activity of untreated tissue		Activity of treated tissue		Activity remaining after treatment of homogenate
	Intact	Disrupted	Intact	Disrupted	
NADase	98 ± 7	100	23 ± 2	21 ± 2	19 ± 2
5'-Nucleotidase	78 ± 6	100	43 ± 4	60 ± 5	50 ± 3
Lactate dehydrogenase	11 ± 2	100	6 ± 1	96 ± 7	48 ± 6

Table 2. *Effect of treatment with the diazonium salt of sulphanilic acid on NADase, 5'-nucleotidase and lactate dehydrogenase activities of intact and disrupted rat thyroid cells (FRT_L) in monolayer culture*

Monolayers were incubated at 37°C with the reagent (5 mM) for 16 min, then washed 10 times with 20 mM-Hepes/NaOH buffer, pH 7.4, containing 0.9% NaCl. Specific activities in disrupted untreated cells (= 100%): NADase, 0.15 µmol/h per mg; 5'-nucleotidase, 1.21 µmol/h per mg; lactate dehydrogenase, 0.47 µmol/h per mg. Values are means ± S.D. for three experiments.

Enzyme	Specific activity (% of that in disrupted untreated cells)			
	Activity of untreated cells		Activity of treated cells	
	Intact	Disrupted	Intact	Disrupted
NADase	100 ± 5	100	1.7 ± 0.2	1.3 ± 0.3
5'-Nucleotidase	69 ± 6	100	13 ± 1.5	39 ± 2
Lactate dehydrogenase	<1	100	<1	97 ± 7

affected. Disruption (homogenization with a Tenbroeck hand-operated homogenizer) of the treated slices did not significantly affect the NADase activity, but a notable increase in 5'-nucleotidase activity was apparent. After disruption of treated slices the lactate dehydrogenase activity was almost completely restored.

A similar series of experiments were performed with functional rat thyroid cells (FRT_L) in culture. To this end monolayer cultures (25 cm² Falcon flasks) were rinsed with KRT buffer and subsequently incubated with 1 ml of KRT buffer containing the appropriate substrates. The flasks were incubated at 37°C, and 0.1 ml samples of medium were removed at specified time intervals for the determination of liberated adenosine or nicotinamide. Under these experimental conditions results were obtained similar to, although more straightforward than, those with the bovine thyroid slices (Table 2). Treatment with the diazonium salt of sulphanilic acid almost completely abolished the NADase activity measured with intact cells. Disruption of the monolayer by the addition of 0.2% sodium deoxycholate and brief sonication did not enhance the activity. The

diazonium salt of sulphanilic acid also severely depressed the 5'-nucleotidase activity of intact thyroid cells. After disruption of the cells it again became apparent that part of the enzyme activity was not associated with the external aspect of the cell membrane. These experiments further support the view that thyroidal NADase is an ecto-enzyme. Although from all the evidence to hand it seems very unlikely, one could, however, still consider the possibility that the diazonium salt of sulphanilic acid is modifying some part of the enzyme that is exposed to the external medium that would perturb the active site located at the cytoplasmic site. To rule out this possibility we studied the effect of the triazine dye Cibacron Blue F3GA and its covalent dextran derivative Blue Dextran. This dye strongly binds to the nucleotide-binding site of NAD⁺- and NADP⁺-linked dehydrogenases (Thompson & Stellwagen, 1976) and kinase (Böhme *et al.*, 1972). Moreover, Cibacron Blue F3GA has been used as a probe for the dinucleotide fold, i.e. a region of supersecondary structure found in the active site of these enzymes (Thompson & Stellwagen, 1976). Schuber & Pascal (1977) also demonstrated that this dye is a powerful inhibitor of solubilized and

partially purified calf spleen NADase, and suggested that this enzyme contains the dinucleotide fold or at least shares large structural similarities. Cibacron Blue F3GA was also able to inhibit strongly the solubilized and partially purified NADase in bovine thyroid (see also under Properties of NADase, below). The inhibition was of the competitive type (K_i 7.5×10^{-6} M). Therefore it seems reasonable to assume that this dye is binding to the nucleotide-binding site of NADase. Microscopic examination indicated that the dye does not appear to penetrate the intact thyroid cell. Addition of Cibacron Blue F3GA (1 mM) to the medium of a monolayer of FRT_L cells completely depressed (>99%) the NADase activity. The high- M_r derivative Blue Dextran at concentrations ranging from 1 to 5 mM (number of chromophores corresponding to a Cibacron Blue concentration of respectively 1 mM and 5 mM) decreased the activity by 70–90%. The decrease in activity on treatment is not due to inhibition of a transport protein, because of the parallel decrease in activity of treated cells and homogenate.

Solubilization and purification of NADase

In thyroid, NADase is tightly bound to the plasma membrane. However, the enzyme was almost completely (>90%) solubilized by detergent extraction with sodium deoxycholate (1.5%, w/v) or with a mixture of Triton X-100 (2%, w/v) and sodium deoxycholate (1%, w/v). After extraction with sodium deoxycholate (1.5%) a 2-fold lower NADase activity was observed. This was unlikely to be due to denaturation, since replacement of sodium deoxycholate (1.5%) by the mixture of Triton X-100 and sodium deoxycholate restored the activity to its original value. Probably the negative charge of sodium deoxycholate adversely affected the interaction of the negatively charged substrate and the NADase-detergent complex. Sodium deoxycholate also decreased the NADase activity associated with the active form of the A protomer of cholera toxin (results not shown). The solubilized thyroid enzyme was further purified by gel filtration on Sephadex G-200. As illustrated in Fig. 3, the NADase activity eluted at a $V_e/V_0 = 1.64$, well separated from the bulk of the proteins. This chromatographic step also resolved the NADase from several other plasma-membrane enzymes, such as alkaline phosphatase and 5'-nucleotidase. The resolution between the protein peak and NADase peak was not always satisfactory when sodium deoxycholate (1.5%) was used as the solubilizing agent. Therefore in the final purification procedure solubilization was always performed with the Triton X-100/sodium deoxycholate mixture.

Further purification with ion-exchange chroma-

Fig. 3. Gel chromatography of NADase and some typical plasma-membrane enzymes on Sephadex G-200. Column dimensions and elution conditions were as described in the Materials and methods section. ●, NADase; ○, alkaline phosphatase; △, 5'-nucleotidase; ■, protein concentration (A_{720} , Lowry method).

tography on CM-cellulose (with a linear salt or pH gradient) or DEAE-cellulose was not successful. The enzyme also failed to bind to 5'-AMP-Sepharose 4B and β -NAD⁺-Sepharose 4B. However, phenyl-Sepharose CL-4B, *Lens culinaris* lectin-Sepharose 4B and Blue Sepharose CL-6B (Fig. 4) were able to retain the NADase. In each case a substantial increase in NADase specific activity was obtained. However, the recovery was rather low and highly variable. The highest recoveries were obtained with Blue Sepharose CL-6B (up to 75%). To this end the NADase peak obtained after the gel filtration was pooled, subjected to acetone precipitation and resuspended in 10 mM-Tris/HCl buffer, pH 7.4. As illustrated in Fig. 4, most of the proteins present in this preparation failed to adsorb on the affinity column, whereas the NADase was almost completely retained. The enzyme was subsequently eluted by applying a linear NaCl gradient. Under these conditions the peak of NADase activity emerged at 0.1 M-NaCl. In order to attain the high recovery mentioned above, it was essential to perform both chromatographic separation and enzyme assays on the same day. Freshly prepared chromatography columns yielded higher recoveries. The results of the final purification scheme are presented in Table 3. With this purification method an overall purification factor of 1940-fold with a yield of 10% could be obtained. The final preparation showed only one major band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, in contrast with NADases purified from other membrane-bound sources, where heterogeneous electrophoretic patterns (Swislocki *et al.*, 1967) were observed. Owing to the high lability of the enzyme after the last

Fig. 4. *Chromatography of NADase on Blue Sepharose CL-6B*

After gel chromatography on Sephadex G-200 fractions containing NADase activity were pooled, subjected to acetone precipitation and after resuspension applied to a Blue Sepharose CL-6B column. NADase was eluted with a linear gradient of NaCl as described in the Materials and methods section. ●, NADase; O, proteins (determined fluorimetrically); —, NaCl gradient.

Table 3. *Purification of NADase from bovine thyroid (100 g of tissue)*
For experimental details see the Materials and methods section.

	Protein (mg)	Total activity ($\mu\text{mol/h}$)	Specific activity ($\mu\text{mol/h per mg}$)	Recovery (%)	Purification factor
Homogenate	15857	634	0.04	100	
M + L + P fraction	786	157	0.2	27	5
M + L + P fraction (after osmotic shock)	366	220	0.6	31	15
Solubilization	321	193	0.6	28	15
Sephadex G-200	25	162	6.5	24	162
Acetone precipitation	17	143	8.4	21	210
Blue Sepharose	0.85	66	77.6	9.6	1940

purification step no activity could be recovered from the gel after gel electrophoresis under non-denaturing conditions. When adding radioiodinated highly purified NADase (step 7 of the purification procedure) to partially purified enzyme (step 6 of the purification procedure) and subsequently subjecting the mixture to gel electrophoresis under non-denaturing conditions a co-migration of radioactivity and NADase activity was observed.

Stability of NADase

The membrane-bound NADase was quite stable. No significant fall in activity occurred after preincubation at pH 7.4 and 37°C for different time intervals. Under these conditions the partially purified enzyme (step 6) showed a half-life time of 6 h. The partially purified enzyme did not show any significant loss of activity when stored for several weeks at -20°C, or at least 6 months in liquid N₂. At 4°C the enzyme loses 25% of its activity in

24 h. After purification on Blue Sepharose CL-6B the enzyme became highly unstable. No activity could be detected upon storage overnight at 4°C, at -20°C or in liquid N₂. All efforts, e.g. the addition of albumin (1 mg/ml), phosphatidylcholine (1 mg/ml), Triton X-100 (1%, w/v), 30–50% (v/v) glycerol, substrate or metal ions, failed to improve the stability of the enzyme.

Properties of NADase.

Membrane-bound as well as the partially purified (step 6) thyroidal NADase display a broad pH optimum with optimal activity around pH 7.2. As a function of substrate concentration no deviation from Michaelis-Menten kinetics is observed. The kinetic parameters for partially purified and membrane-bound NADase are similar ($K_{m, \text{purified}} = 0.42 \text{ mM}$; $K_{m, \text{membrane-bound}} = 0.35 \text{ mM}$). For NADP⁺ a K_m value of 0.62 mM was found. The partially purified enzyme (step 6 of the purification procedure) splits β -NAD⁺, β -NADP⁺, β -NADH and α -NAD⁺ at

Table 4. *Transglycosidase activities of bovine thyroid and rat liver NADases*
For experimental details see the Materials and Methods section.

Source	NADase specific activity ($\mu\text{mol/h per mg}$)	Transglycosidase specific activity (nmol/h per mg)
Bovine thyroid M+L fraction	0.2	8.1
Bovine thyroid enzyme (peak fraction, Sephadex G-200)	7.6	383.0
Rat liver microsomal fraction	0.7	50.0

rates in the relative proportions 1.0:0.75:<0.02:<0.02. The reaction products nicotinamide and ADP-ribose both inhibit the NADase activity. According to the Lineweaver-Burk plots obtained with partially purified NADase, ADP-ribose is a competitive inhibitor with a $K_i = 2.7\text{ mM}$, and nicotinamide acts as a non-competitive inhibitor with a $K_i = 1.9\text{ mM}$. This is consistent with an Ordered Uni Bi reaction with a sequential release of the products from the enzyme during hydrolysis of NAD⁺, nicotinamide being released first and ADP-ribose last (Schuber *et al.*, 1976).

The thyroidal NADase also displays transglycosidase activity, i.e. it is able to catalyse the pyridine-base-exchange reaction. The ratio of NADase to transglycosidase activity is comparable with that found for rat liver membrane-bound NADase under our experimental conditions. As shown in Table 4, purification does not affect this ratio. This indicates that the reactivity of water near the active site of membrane-bound and isolated NADase is very similar.

Cibacron Blue F3GA and Blue Dextran are potent inhibitors of the partially purified as well as of the membrane-bound and of intact cell-bound NADase. The inhibition is of the competitive type ($K_{i, \text{purified}} = 7.5\text{ }\mu\text{M}$). Inhibition potencies decrease in the same order. Probably charge effect and/or steric hindrance are responsible for this phenomenon.

As summarized in Table 5, ATP, ADP, AMP and cyclic AMP did not inhibit the hydrolysis of NAD⁺ even when these compounds were added to the reaction mixture at concentrations up to 1 mM. HgCl_2 is a potent inhibitor, but *p*-chloromercuribenzoate does not affect the activity. Dithiothreitol only weakly inhibits the activity, which is in contrast with the observation by Apitz *et al.* (1971), who reported a marked inhibition for the pig brain enzyme. ADP-ribose acceptors such as L-arginine methyl ester and imidazole also inhibit the enzymic activity. The enzyme does not show any ADP-ribosyltransferase activity. Bovine thyroid histones exert a stimulatory effect (1.5-fold) on the thyroidal enzyme.

Although there was a marked overall increase in

Table 5. *Effectors of NADase*

Effectors were added to the standard assay system containing purified NADase (step 6). All additions to solutions were adjusted to pH 7.4. For experimental details see the Materials and methods section. Values are the means for three separate experiments.

Effector	Concentration	Activity (%)
None	—	100
Nicotinamide	1 mM	64
ADP-ribose	5 mM	74
N-Ethyl nicotinamide	1 mM	100
N-Diethyl nicotinamide	1 mM	95
3-Acetylpyridine	10 mM	77
	1 mM	98
AMP	1 mM	100
ADP	1 mM	100
ATP	1 mM	100
Cyclic AMP	1 mM	100
Cibacron Blue F3GA	10 μM	52
β -NADP ⁺	1 mM	54
	0.1 mM	83
HgCl_2	10 mM	3
	1 mM	13
	0.1 mM	94
<i>p</i> -Chloromercuribenzoic acid	0.1 mM	100
Dithiothreitol	1 mM	80
L-Arginine methyl ester	0.1 M	49
Imidazole	0.4 M	21
	0.1 M	39
Thyrotropin	5 μM	100
	1 μM	100
Total thyroid histones	1 mg/ml	145
Phosphatidylinositol	1 mg/ml	67
Phosphatidylserine	1 mg/ml	73
Phosphatidylethanolamine	1 mg/ml	97
Phosphatidylcholine	1 mg/ml	123

the specific activity of NADase during the purification scheme applied, an appreciable loss of overall enzyme activity (Table 3), especially during the last purification step, was observed. Therefore an attempt was made to increase the enzymic activity of purified enzyme by the addition of phospholipids. Addition of dispersions of different phospholipids to the partially purified enzyme did

not produce any significant increase in enzyme activity. Anionic phospholipids such as phosphatidylinositol and phosphatidylserine decreased the enzyme activity, though the zwitterionic phospholipid phosphatidylcholine was slightly activating (Table 5). From these data it seems that phospholipids are not essential for enzymic activity. This was further supported by the observation that evaluation of the thyroidal membrane-bound NADase activity as a function of temperature did not reveal any break or discontinuity in the Arrhenius plot. Therefore it appears that the catalytic part of this enzyme has no functional relationship with its lipid environment.

The estimation of the M_r of the purified enzyme was carried out on a Sephadex column (2.5 cm $\times$ 180 cm) equilibrated with 10 mM-Tris/HCl buffer, pH 7.4, containing 1% Triton X-100 and 0.2% sodium deoxycholate and calibrated with bovine serum albumin (M_r 67 000), fructose biphosphate aldolase (M_r 147 000), globulin (M_r 160 000) and catalase (M_r 240 000). A linear relationship was obtained for the elution volumes of the standard proteins and log M_r values. Under these conditions the enzyme showed an M_r of 120 000 ($\pm$ 2000).

Discussion

Only scant attention has been devoted to the subcellular localization of NADases in mammalian tissues. In most tissues investigated the enzyme appears to be tightly bound to microsomal particles. It should be stressed, however, that in most of these preparations an enrichment of 5'-nucleotidase was also observed. Ultrastructural studies by Bock *et al.* (1971) revealed that the enzyme in rat liver is distributed over both endoplasmic reticulum and plasma membranes. Amar-Costesec & Beaufay (1977), however, demonstrated that the bulk of NADase in rat liver is associated with a single subcellular entity originating from the pericellular membrane. Also, in calf spleen the bulk of this enzyme appeared to be located in plasma membranes (Muller & Schuber, 1980). From all the evidence presented in this report it is concluded that the thyroidal NADase is predominantly located in the plasma membrane and that most if not all of the enzyme (catalytic site) is accessible to extracellular NAD^+ . These observations, in conjunction with the detergent requirement for solubilization, clearly demonstrate that thyroidal NADase is an integral protein of the plasma membrane with its active site located at the outside surface of the cell. Therefore thyroid NADase meets the criteria of an authentic ecto-enzyme (Stanley *et al.*, 1982). In contrast with NADase, a rather large proportion (25%) of 5'-nucleotidase, which has been shown to be an ecto-

enzyme in several tissues studied (De Pierre & Karnovsky, 1973), is present inside the cell. This could indicate that NADase is unlikely to be internalized during membrane recycling. An alternative explanation would be that the internalized portion of NADase is inactive, turning into an active state upon transfer to the surface of the cell. When applying a similar experimental approach to other cell types such as human fibroblasts and mouse myeloma cells (results not shown) we found that in both cases most of NADase (>95%) was associated with the outer aspect of the cell surface. With rat tissue slices, however, only 50% of the activity was accessible to extracellular NAD^+ . Cibacron Blue F3GA (1 mM) strongly depressed (decrease of activity by 85%) the NADase activity of intact rat liver slices. On the other hand the diazonium salt of sulphanilic acid did not significantly affect this activity. However, under our experimental conditions this reagent also failed to inactivate the 5'-nucleotidase activity associated with intact rat liver slices. Data taken from the literature suggest that NADases from erythrocytes (Alivisatos *et al.*, 1974), Ehrlich ascites cells (Bock *et al.*, 1968), macrophages (Artman & Seeley, 1979a), liver (Liersch *et al.*, 1971) and spleen (Muller & Schuber, 1980) are also associated with the cell surface. That the active site is facing the extracellular medium has been clearly established only for bovine erythrocytes (Pekala & Anderson, 1978) and mouse macrophages (Artman & Seeley, 1979b). Since the present work was completed we also became aware of data showing that the NADase of calf spleen is an ecto-enzyme (Muller *et al.*, 1983). These data, taken together with our results, suggest that NADases are ecto-enzymes in several if not in most cell types. In contrast with the NADases from bacterial origin, which are soluble, all mammalian NADases except the one present in seminal plasma (Yuan & Anderson, 1971) are membrane-bound. It may be noted that 5'-nucleotidase is an ecto-enzyme in most tissues studied except again in seminal plasma, where it is present in a soluble form (Levin & Bodansky, 1966). The thyroidal enzyme could almost completely be solubilized by detergent extraction. This is in contrast with reports (Kaplan, 1955) that particulate NADases from mammalian sources would be resistant to solubilization by deoxycholate, digitonin etc. The solubilized enzyme was purified by gel filtration and affinity chromatography. The overall procedure resulted in a 1940-fold purification (specific activity: 78 μmol of nicotinamide released/h per mg of protein). This value lies between those reported for other purified NADases [calf spleen, 420 μmol /h per mg (Schuber & Travo, 1976); rat liver, 588 μmol /h per mg (Bock *et al.*, 1971); Ehrlich ascites cells, 13.8 μmol /h per

mg (Green & Bodansky, 1965)]. Compared with values reported for other tissues (ranging from 25000 to 90000) the thyroidal NADase has a rather high M_r . However, with most membrane-bound NADases studied solubilization was achieved by treatment with trypsin or crude pig pancreatic lipase (Stathakos & Wallenfels, 1966; Green & Bodansky, 1965). Under these conditions probably only tryptic fragments were obtained (Schuber & Travo, 1976).

The thyroidal NADase appears to be a glycoprotein containing sialic acid. This follows from the observation of specific binding (reversal of binding by α -methyl mannoside) to *Lens culinaris* lectin-Sepharose 4B and a perturbation of the isoelectric-focusing pattern (shift to higher pI values) on treatment with sialidase (*Vibrio cholerae*) (results not shown).

Apart from its hydrophilic nature the protein has a hydrophobic domain probably providing the anchor by which the enzyme is linked to the membrane. This follows mainly from the observation that the partially purified enzyme strongly adsorbs on a phenyl-Sepharose CL-4B column and that phospholipids are able to perturb the catalytic activity. In its sensitivity to product inhibition, specificity towards NAD⁺ and NADP⁺ and intrinsic transglycosidase activity the thyroidal NADase resembles that of other mammalian tissues.

Several points should be mentioned in conclusion. (i) Previous studies have shown that TSH increases the nicotinamide nucleotide content of normal rat thyroids *in vivo* (Maayan & Rosenberg, 1966). Maayan (1964) considered the possibility that this could be due to an effect on NADase. Our results, however, do not favour this hypothesis: TSH has no effect on NADase, and if an effect should occur *in vivo* it cannot be confined to the interior aspect of the thyroid cell. (ii) It has been suggested that the biochemical role of NADases is the regulation of certain metabolic processes through a control of the quantity of NAD⁺ available to the NAD⁺-dependent enzymes (Kaplan, 1966). However, there is no direct relation between NADase activity and NAD⁺ concentrations in tissues in different states such as carcinoma, starvation, response to certain drugs etc. (Apitz *et al.*, 1971; Green & Dobryanski, 1967; Clark & Pinder, 1969). According to Maayan (1964), this enzyme may also influence the thyroidal deiodination of iodotyrosines by affecting the availability of NADPH. The inhibition of NADase and the parallel enhancement of deiodinating activity of normal rat thyroid glands upon the addition of 75mM-nicotinamide was used to demonstrate this point. Again, the extracellular localization of the thyroidal NADase active site

makes it unlikely to be involved in controlling the intracellular NADP⁺ concentration, unless there should exist some kind of mechanism making the substrate (NADP⁺) available to the extracellular space. On the other hand the demonstration that the thyroidal NADase is an ecto-enzyme makes it a good candidate for participation in extracellular events such as hormone-receptor interaction. (iii) As an ecto-enzyme NADase might also be involved in mechanisms that trigger cell growth and differentiation (Stanley *et al.*, 1982). The different NADase activity values observed with functional (FRT_L) and non-functional (FRT) rat thyroid cells in culture support this presumption. (iv) A role in the mechanism of hormone message transmission and agonist (TSH)-induced refractoriness is also not excluded. Several lines of evidence point in this direction. Functional rat thyroid cells (FRT_L), chronically stimulated with TSH, have high NADase activity and low mono-ADP-ribosyltransferase activity. These cells are desensitized to further addition of TSH, i.e. more TSH does not affect cyclic AMP concentrations (Vitti *et al.*, 1982). Studies with intact human thyroid adenoma cells (19 HT) have shown that prevention of desensitization was observed on addition of artificial mono-ADP-ribose acceptors such as arginine and arginine methyl ester (Filetti *et al.*, 1980). Furthermore, nicotinamide and 1-methylnicotinamide, which are powerful inhibitors of mono-ADP-ribosyltransferase and NADase, similarly prevented TSH desensitization in cultured human thyroid cells (Filetti *et al.*, 1981a). Other experiments (Filetti *et al.*, 1981b) have shown that cellular interactions of cultured thyroid cells modulate the TSH responsiveness, i.e. thyroid cells in confluent culture do not show any desensitization. These experiments suggest that contact inhibition among confluent cells could account for an escape from TSH desensitization. (v) Finally, from a methodological point of view the enzyme can be used as an appropriate marker for thyroidal plasma membranes. It also provides a suitable antigen for studying membrane-related phenomena such as recycling by immunological techniques.

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