AMPHIPATHIC PROPERTIES OF CALF SPLEEN NAD GLYCOHYDROLASE

Francis SCHUBER, Hélène MULLER and Isabelle SCHENHERR
Institut de Botanique de l'Université Louis Pasteur (LA 31), 28, rue Goethe, 67000 Strasbourg, France

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1. Introduction

Mammalian NAD glycohydrolases (EC 3.2.2.6) have been isolated from a variety of tissues and in most cases the highest enzyme activity was found associated with microsomes [1–3]. Solubilization of several membrane-bound NAD glycohydrolases was achieved with steapsin or proteases and some of these enzymes, including calf spleen NADase [4], could be highly purified by use of standard techniques for soluble protein fractionation [5–7]. The reaction mechanism of NAD hydrolysis, catalyzed by NAD glycohydrolase, has been mostly studied with soluble [8] or solubilized forms of the enzyme, e.g., pig brain [9,10] and calf spleen NADases [11,12]. However, it is well known that the properties of a membrane-bound enzyme often differ from those of the same enzyme solubilized, as studied in homogeneous medium [13]. Catalytic properties of a bound enzyme can be influenced by its microenvironment and by the existence of interactions with other membrane constituents such as phospholipids [13]. The activity of some membrane-bound NAD glycohydrolases has been shown to be influenced by alterations of the lipid-phase structure [14,15]. Interestingly, in some pathologies, e.g., tuberculosis, a soluble NADase form could be detected in infected organs [16].

In order to delineate the contribution of the membrane environment to calf spleen NAD glycohydrolase activity, we have compared the catalytic properties of the membrane-bound (microsomal) and the hydrosoluble forms of the enzyme [17]. Such a study revealed the need for a better knowledge of the mode of association of this class of enzymes with membranes. Here we report evidence that calf spleen NADase presents characteristics of an amphipathic protein, i.e., the enzyme is composed of a hydrophilic region containing the catalytic domain and of a hydrophobic part probably responsible for its association with membranes.

2. Materials and methods

2.1. *Chemicals*

Emulphogene BC-720 was from GAF, Sepharose 6B-CL and Sephacryl S-200 from Pharmacia. The alkyl-agaroses kit was purchased from Miles Biochem. and proteinase K from Merck. All other biochemicals and products were from Sigma Chem. Co. Solubilized calf spleen NADase was prepared and purified as in [4].

2.2. *Membrane fractions*

Fresh calf spleens were homogenized, at 4°C, in 5 vol. 0.3 M sucrose with a Teflon–glass homogenizer. The post-mitochondrial supernatant, obtained at 150 000 × *g*. min, was centrifuged at 100 000 × *g* for 1 h. Microsomal pellets were washed in 0.15 M Tris–HCl (pH 8.0) and finally resuspended in 50 mM sodium phosphate buffer (pH 7.4). Proteins were determined by a modified Lowry method [18].

2.3. *Solubilization experiments*

The solubilization agents (ions, enzymes and detergents) were added to the microsomal suspensions (5–10 mg membrane protein/ml) at the concentrations in section 3. The final medium volume was from 1–2 ml. After the incubation, at 25°C, the mixtures were centrifuged at 100 000 × *g* for 1 h. NADase activity was assayed [4] in the supernatants (solubilized enzyme) and in the resuspended pellets. As a control, the membrane-bound NADase was incubated under similar conditions, in the absence of the solubilizing agent.

3. Results

Several classes of membrane-bound enzymes exist, e.g., peripherally bound and integral enzymes, which differ by the nature of the interactions with membranes and by their behaviour in aqueous solutions. These enzymes can be distinguished by the use of different solubilization methods [13,19,20]. Microsomal calf spleen NAD glycohydrolase was therefore subjected to a series of solubilizing agents.

3.1. *Action of ions*

High ionic strength (e.g., 1 M KCl), ultrasonication at low ionic strength (5 mM KCl), pH changes or chelating agents (e.g., 50 mM EDTA) did not dissociate NADase from the membrane. Destabilization of membranes with chaotropic ions ($\leqslant$4 M $NaClO_4$ or NaSCN), shown [21–23] to extract membrane-proteins, failed to solubilize the enzyme in appreciable amounts. It seems likely that calf spleen NADase is more firmly bound to membranes than peripheral proteins.

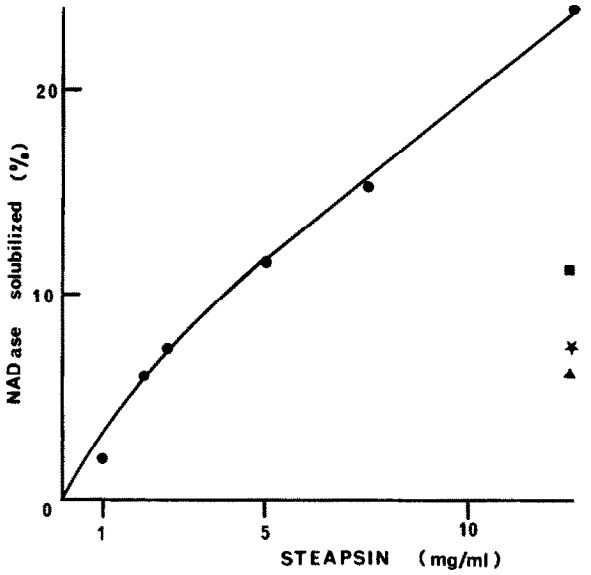

Fig.1. Solubilization of calf spleen microsomal NAD glycohydrolase with steapsin. Microsomes (5 mg protein; 1.3 unit NADase) were incubated with varying amounts of steapsin in a 50 mM sodium phosphate buffer (pH 7.4) containing 0.01 M $CaCl_2$ (final vol. 1 ml). After 1 h at 25°C the mixtures were centrifuged and enzyme activity was assayed in the supernatants. In some experiments steapsin was preincubated (30 min, at 25°C) with 1 (■) or 5 (▲) mg soybean trypsin inhibitor and with 0.1 mM phenylmethylsulfonyl fluoride (*). The results are expressed as % of original NADase activity.

3.2. *Solubilization with steapsin*

A hydrosoluble form of NADase was obtained by treatment of calf spleen acetone powders with crude porcine pancreatic lipase (steapsin) [24,4]. Solubilization of microsomal NADase required high amounts of steapsin (fig.1). The solubilization was inhibited by soybean trypsin inhibitor, and was also largely prevented when steapsin was pretreated (according to [25]) with 0.1 mM phenylmethylsulfonylfluoride. It appears that a serine-protease contaminant is an important factor in the solubilization of NADase by steapsin; a similar observation was made with the solubilization of NADPH-cytochrome *c* reductase [26].

3.3. *Action of proteases and phospholipases*

Trypsin and α-chymotrypsin (e.g., 100 μg protease/mg membrane protein, at 25°C, for 1 h incubation) were unable to mimic the solubilization obtained with steapsin. Several other proteases, including papain, elastase, crude pancreatic protease, pronase and thermolysin, did not, under optimal conditions, yield satisfactory solubilizations (at best ~10%). Membrane-bound NADase proved to be fairly resistant to proteolytic attack, only proteinase K, albeit at high concentrations, was able to inactivate the enzyme in appreciable amounts; e.g., ~60% of NADase activity was lost after incubation (1 h, at 37°C) with 200 μg proteinase K/mg membrane protein. Progressive membrane destructuring with deoxycholate resulted in increased NADase inactivation; e.g., ~90% of NADase activity was lost, under similar conditions, when 0.075% (w/v) detergent was added to the incubation medium. This observation might indicate that the enzyme became more exposed to the action of the protease.

Phospholipase C and A_2, which have been used in several instances for solubilizing membrane-bound enzymes [19], did not, under optimal conditions, significantly release NADase from the microsomes. Concomitantly we observed no activation or inactivation of the enzyme, which confirms that calf spleen NADase has probably no stringent phospholipid environment requirement for its activity. The solubilization yield obtained with steapsin could be doubled by the addition of phospholipase A_2, suggesting that a combined action of a phospholipase and a protease is necessary to obtain a water-soluble form of NADase. When assayed with egg yolk as substrate [27], the steapsin used in this study presented a phospholipase A_2 activity of ~1 unit/mg.

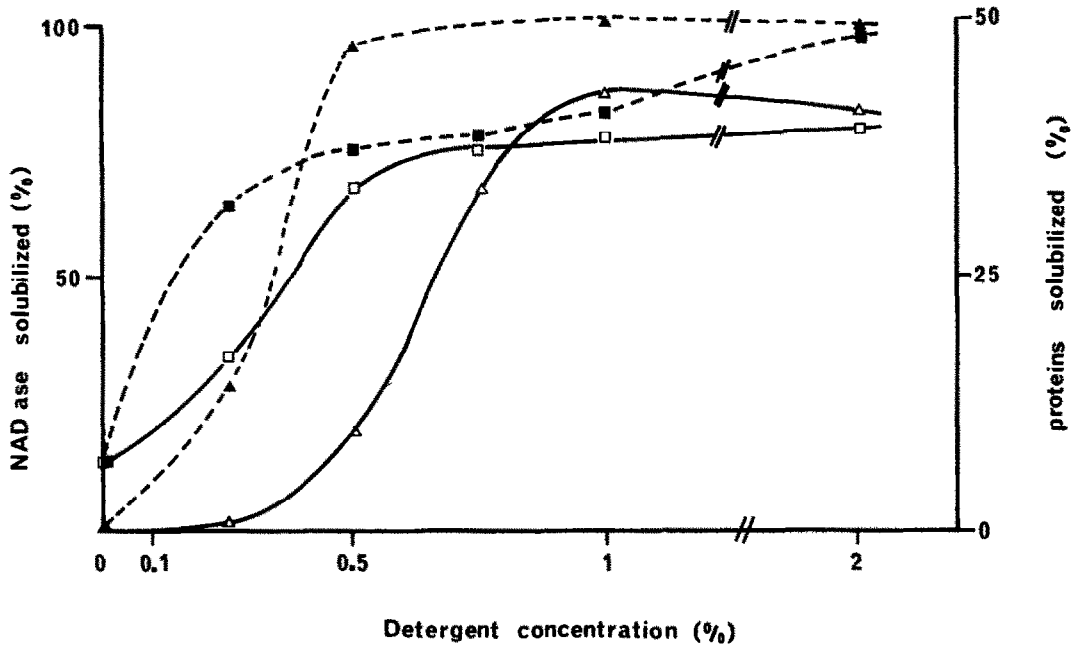

Fig.2. Comparative solubilization of membrane-bound NAD glycohydrolase and protein by cholate and emulphogene. Microsomes (1.6 mg protein/ml) were incubated with varying cholate (open symbols) and emulphogene (closed symbols) concentrations (w/v) in a 50 mM Tris–HCl buffer (pH 7.5) (final vol. 1 ml). After 30 min at 37°C, the mixtures were centrifuged at 100 000 × *g* for 1 h. NADase activity (△, ▲) and protein concentration (□, ■) were determined in the supernatants. The results are expressed as % of original values.

3.4. *Solubilization of NADase with detergents*

The foregoing results which indicate that NADase is a firmly membrane-bound protein and the possibility to isolate a hydrosoluble form of the enzyme by action of steapsin [4], are reminiscent of the characteristics of some typical amphipathic proteins. Such proteins, e.g., cytochrome b_5 [28], have a hydrosoluble catalytic domain which can be split off and are anchored to the membrane by a hydrophobic sequence. It was of interest therefore to obtain an intact form of calf spleen NADase. For this purpose we have tried to solubilize the enzyme with detergents.

Calf spleen NADase was found to be severely inhibited by deoxycholate and taurocholate (e.g., 50% inhibition at 0.05% and 0.2% (w/v) of these detergents, respectively). This inhibition was fully reversible on dilution and was much less marked with cholate. Deoxycholate and the non-ionic detergents Triton X-100 and Emulphogene BC-720, which had no inhibitory action on the enzyme activity, solubilized membrane-bound NADase in excellent yields. This confirms reports that bovine spleen NADase is resistant to solubilization by deoxycholate [29]. The data obtained in fig.2. show that, under the experimental conditions used, the detergents solubilized total protein somewhat faster than NADase activity. The solubilized enzyme was not sedimented at 100 000 × *g* for 1 h and penetrated into Sepharose 6B gels. When a deoxycholate-solubilized enzyme preparation was dialyzed against a buffer lacking detergent, the NADase did not remain in solution. The aggregated enzyme could be resolubilized with a detergent containing medium.

3.5. *Characterization of the detergent solubilized NADase*

The detergent form of NADase presented an app. mol. wt 90 000 as estimated by permeation chromatography, in the presence of 0.1% emulphogene, on calibrated Sepharose 6B and Sephacryl S-200 columns. This value is in marked contrast with the molecular weight of the hydrosoluble form of the enzyme: M_r = 24 000 [4], and probably also accounts for the binding of detergent molecules (micellar form). Contrary to the membrane-bound NADase, the detergent-solubilized enzyme was, under similar

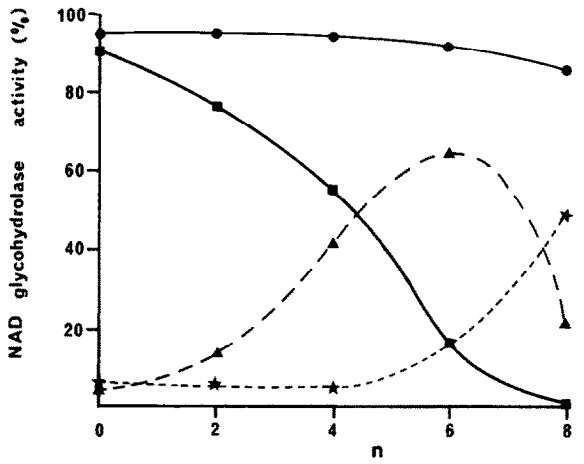

Fig.3. Hydrophobic chromatography of hydrosoluble and detergent-solubilized forms of NAD glycohydrolase. Samples containing ~0.4 unit of hydrosoluble (•) and detergent-solubilized (▪) NADase (1.5 mg protein) were applied, at 4°C, to columns containing 1 ml alkyl-agarose (agarose–c_n with $0 \leq n \leq 8$) gels, pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.4). Non-adsorbed proteins were washed off with the same buffer (5 ml) and the fractions were tested for NADase activity. Bound detergent-solubilized NADase was eluted with 5 ml fractions of the phosphate buffer containing successively 0.1% (▲) and 0.5% (★) emulphogene (w/v). The results are expressed as % of total activity recovered.

experimental conditions, very rapidly inactivated by trypsin. The hydrophobic characteristics of this new NADase form were further assessed by hydrophobic chromatography by the method in [30]. Detergent-form obtained by solubilization with cholate and freed from excess detergent by passage on Sephadex G-50, was strongly adsorbed by alkyl-agarose columns (fig.3); this is in marked contrast with the failure of hydrosoluble NADase to be significantly retarded by the same columns. Dissociation of detergent NADase form from the columns required detergent solutions (fig.3).

4. Discussion

Calf spleen NAD glycohydrolase, which we have shown to be tightly bound to membranes, is most likely an integral protein. Since we have demonstrated that the active site of NADase is located on the exterior side of the microsomal vesicles (H.M., F.S., submitted), it is excluded that the marked resistance of the bound enzyme to proteolytic inactivation (which is not an intrinsic property of the enzyme) is caused by a hindered approach of the protease due to the inherent sidedness of membrane vesicles. This resistance, and the permissive effect of detergent (table 1), rather indicate that the NADase is probably somewhat buried in the phospholipid bilayer. A similar conclusion was reached in [31] for rat liver NADase; it was observed that antibodies raised against the solubilized-hydrosoluble form of the enzyme inhibited much less the microsomal form of the enzyme [31].

A hydrosoluble form of NADase (M_r = 24 000) could be obtained by treatment of calf spleen microsomes with steapsin. Our results indicate that this solubilization probably results from a combined phospholipase and protease action, which might be rendered necessary by the mode of association of the enzyme with membranes. The catalytic properties of membrane-bound and hydrosoluble NAD glycohydrolase are very similar [17]. One can therefore assume that the hydrosoluble form of NADase, obtained by proteolysis, is the catalytically active domain of the enzyme, though it is not the molecular species present in vivo. The lack of hydrophobic properties of hydrosoluble NADase (fig.3), indicates that this form of the enzyme probably lacks the area responsible for membrane attachment. It follows that an intact form of NAD glycohydrolase must possess an additional protein sequence which enables the enzyme to interact strongly with membranes.

The action of ionic and non-ionic detergents on microsomes allowed the characterization of a new solubilized catalytically active form of calf spleen NAD glycohydrolase, which is presumably the intact form of the enzyme. The detergent-solubilized enzyme presents, compared to the hydrosoluble NADase, distinct molecular properties and most important it is a hydrophobic protein which needs a micellar environment to remain in solution. Apparently the hypothetical additional sequence, lacking in the hydrosoluble NADase, confers hydrophobicity on the whole enzyme molecule and explains a higher apparent molecular weight. The detergent-solubilized NADase is strongly adsorbed by alkyl-agaroses, an observation which hints at the functional role of the hydrophobic sequence of this enzyme form. We propose that calf spleen microsomal NAD glycohydrolase is an amphipathic protein. The NADase protein is composed of a hydrophilic sequence, i.e., hydrosoluble NADase form, containing the catalytic domain, which might

be covalently linked to a hydrophobic region. This latter sequence, which can be split off by steapsin, because of its association with, e.g., phospholipids would allow the anchoring of the enzyme to membranes and the orientation of its active site at the interface. The purification and characterization of the detergent form of NAD glycohydrolase are in progress. Its proteolytic conversion into the hydrosoluble form and incorporation into liposomes will be the next steps in order to substantiate further our hypothesis.

The mode of association of calf spleen NAD glycohydrolase with membranes, as discussed here, might be rather general since several membrane-bound NADases could be solubilized with steapsin or proteases (cf. section 1) and with detergents [15, 31–33]. Calf spleen NADase appears to belong to an expanding family of membrane-bound enzymes which are anchored to membranes by a hydrophobic tail, their functional site being associated with a cleavable hydrophilic domain; e.g., microsomal mixed-function oxidase constituents [34] and intestinal brush border pericellular hydrolases [35,36].

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