Asymmetric reassociation of calf spleen NAD⁺ glycohydrolase into liposomes

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NAD⁺ glycohydrolase (NAD⁺ nucleosidase, EC 3.2.2.6) can be solubilized from calf spleen microsomes (microsomal fractions) by steapsin or by detergents to yield respectively a hydrophilic (i.e. water-soluble) and a hydrophobic form of the enzyme. The detergent-solubilized enzyme was successfully reassociated into phosphatidylcholine liposomes either by a cholate-dialysis or by a gel-filtration procedure. In both cases the incorporation of NAD⁺ glycohydrolase was found to be completely asymmetric, i.e. the active site of the enzyme was exposed only at the outer surface of the vesicles. By contrast, as judged by flotation experiments, the hydrophilic form of NAD⁺ glycohydrolase could not be reassociated into liposomes. These results are in agreement with the hypothesis that calf spleen NAD⁺ glycohydrolase is an amphipathic protein. When incorporated into large unilamellar vesicles composed of phosphatidylcholine, NAD⁺ glycohydrolase was not found to catalyse vectorial transfer of NAD⁺ by transglycosidation with nicotinamide as acceptor.

INTRODUCTION

Mammalian NAD⁺ glycohydrolases [NAD(P)⁺ nucleosidases, EC 3.2.2.5/6] are mostly membrane-bound enzymes (Pekala & Anderson, 1982) which, in several cell types, have been shown to be predominantly associated with the plasma membrane (Amar-Costescu & Beaufay, 1977; Muller & Schuber, 1980), their active site being located at the outer surface of the cells (Artman & Seeley, 1979; Muller et al., 1983; De Wolf et al., 1985). Very little is known on the mode of association of those ecto-enzymes with the cell membranes. We have shown that calf spleen NAD⁺ glycohydrolase is an integral membrane-bound protein that can be isolated from microsomes (microsomal fractions) in two catalytically active forms (Schuber et al., 1980): (i) a hydrophilic (i.e. water-soluble) form obtained by treatment of the membranes with steapsin (crude pancreatic lipase), the solubilization resulting from a combined proteolytic and (phospho)lipolytic activity (Schuber et al., 1980), and (ii) a detergent-solubilized form, characterized by its hydrophobicity and which we have recently purified (Muller, 1984). This behaviour is typical of amphipathic proteins, which are composed of a hydrophilic domain, containing, in the case of enzymes, the active site, and of a hydrophobic domain, responsible for the anchoring of the protein to the membranes, that can be cleaved by proteolytic treatment (Spatz & Strittmatter, 1973; Maroux et al., 1977; Hauser & Semenza, 1983; Hughey & Curthoys, 1976). In order to demonstrate further the amphipathic nature of calf spleen NAD⁺ glycohydrolase, we have now compared the ability of the two purified forms of the enzyme to reassociate into artificial phospholipid membranes. Only the detergent-solubilized enzyme could be incorporated into phosphatidylcholine vesicles obtained by either a cholate-dialysis (Kagawa & Racker, 1971) or a gel-filtration (Enoch & Strittmatter, 1979) procedure. Moreover, these reassociations were highly asymmetric, i.e. the catalytically active domain was located outside the liposomes, mimicking the orientation exhibited in native membranes.

MATERIALS AND METHODS

Chemicals

Emulphogene BC-720 was from GAF (France) (Louvres, France), Triton X-100 from Sigma Chemical Co. and sodium cholate from Merck. Sepharose 4B-CL and Sephadex G-75 were purchased from Pharmacia. Phosphatidylcholine was extracted from egg yolk and purified as described by Nielsen (1980) and d,l-[14C]palmitoyl-L α-phosphatidylcholine (114 Ci/mol) was from Amersham. [6(6)-Carboxyfluorescine (Eastman Kodak Co.) was purified as described by Ralston et al. (1981). 1,N⁶-Etheno-NAD⁺ was synthesized as described by Muller et al. (1983). Metrizamide was from Nyegaard, formate dehydrogenase from Boehringer and steapsin from Sigma Chemical Co.

Preparation of detergent-solubilized NAD⁺ glycohydrolase

The solubilization and purification of this form of the enzyme has been published elsewhere (Muller, 1984), and will only be described briefly. Calf spleen microsomes were prepared (Muller & Schuber, 1980) and resuspended (15 mg of protein/ml) in 10 mm-Tris/HCl buffer, pH 8.1, containing 0.1 mm-phenylmethylsulphonyl fluoride and 0.1 mm-EDTA. Emulphogene was then added (final concn. 3%, w/v) and the solubilized enzyme, obtained in the supernatant after centrifugation for 60 min at 105000 g, was purified by successive ion-exchange-chromatography steps on DEAE-cellulose (negative step), CM-Sepharose 6B (50 mm-sodium acetate, pH 5.0, 0.2% Emulphogene and a linear NaCl gradient from 0 to 0.3 m). Further purification and enzyme concentration...
was achieved by chromatography on hydroxyapatite. The latter step was also convenient for detergent-exchange purposes. At this stage a specific activity of about 20 units/mg of protein was routinely obtained, corresponding to a 60-fold and 600-fold purification relative to the microsomes and to whole spleen homogenate respectively.

NAD⁺ glycohydrolase activity was measured by a standard discontinuous assay (Schuber & Travo, 1976) or by a fluorimetric method using 1, N⁶-etheno-NAD⁺ as substrate (Muller et al., 1983). Proteins were determined by a modified Lowry method (Schacterle & Pollack, 1973), with bovine serum albumin as standard.

**Preparation of the hydrophilic (water soluble) form of NAD⁺ glycohydrolase**

Calf spleen microsomes in 10 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-CaCl₂ (15 mg of protein/ml) were treated for 90 min at 30 °C with steapsin (a 2-fold excess, w/w, was required to achieve a 50% solubilization). At the end of the incubation phenylmethylsulphonyl fluoride (final concn. 1 mM) was added to the mixture, which was cooled at 4 °C and centrifuged for 60 min at 100000 g. The supernatant was then immediately chromatographed on a DEAE-cellulose column and further purified as described previously (Schuber & Travo, 1976).

**Incorporation of NAD⁺ glycohydrolase into phospholipid vesicles**

The first method of liposome preparation involved a cholate-dialysis procedure (Kagawa & Racker, 1971; Bauman et al., 1980). Egg phosphatidylcholine (1 mg), trace-labelled with [¹⁴C]phosphatidylcholine (5 x 10⁶ d.p.m.), in 0.5 ml of diethyl ether was dried under a stream of N₂. The lipid film was then dispersed in 0.5 ml of 10 mM-potassium phosphate buffer, pH 7.4, containing 140 mM-NaCl (buffer A) and 1% (w/v) sodium cholate. To this mixture 1 ml of NAD⁺ glycohydrolase preparation was added, i.e. respectively 80 µg of detergent-solubilized enzyme or 18 µg of the water-soluble enzyme in 10 mM-potassium phosphate buffer, pH 7.4, and 0.4% (w/v) sodium cholate. The final mixture was then sonicated for 1 min at 25 °C under N₂ in a bath-type sonicator and dialysed at 4 °C for 60 h against 2 x 2 litres of buffer A. The amount of enzyme associated with the vesicles was then determined by using a flotation centrifugation in a linear sucrose gradient (see the legend to Fig. 1 below).

In an alternative procedure, detergent-solubilized enzyme was reassociated into large unilamellar vesicles obtained by the gel-filtration method of Enoch & Strittmatter (1979; method II). A dried film of egg phosphatidylcholine (8 mg) was dispersed in 1 ml of 10 mM-potassium phosphate buffer, pH 7.4, containing 100 mM-NaCl (buffer B), 0.4% (w/v) sodium cholate and 15 µg of purified enzyme. At this stage, compounds to be entrapped were added, e.g. 100 mM-5(6)-carboxyfluorescein to replace NaCl. After a sonication of 1 min under N₂ at 25 °C, the suspension was filtered on a 30 cm x 1.2 cm column of Sephadex G-75, and the elution (1 ml/min) was performed with the same buffer. The liposomes were then isolated and concentrated by flotation in a discontinuous Metrizamide gradient (Papahadjiopoulos et al., 1981). Samples of the liposome preparation (1.5 ml) were adjusted to 10% (w/v) metrizamide and overlaid with 3 ml of 5% metrizamide topped with a layer of 0.5 ml of buffer B. After centrifugation for 30 min at 150000 g, the liposomes band was collected from the upper interface.

A similar procedure was adopted to prepare liposomes that encapsulate formate dehydrogenase and incorporate NAD⁺ glycohydrolase in their bilayer. Briefly, a mixture containing detergent-solubilized NAD⁺ glycohydrolase (1.2 units), formate dehydrogenase (3.2 units) and egg phosphatidylcholine (20 µmol) in 1 ml of 10 mM-potassium phosphate buffer, pH 7.4, containing 100 mM-NaCl, 10 mM-sodium formate (buffer C) and 0.43% (w/v) sodium cholate, was filtered on a 25 cm x 1.8 cm Sepharose 4B-CL column, which had previously been coated with small unilamellar vesicles composed of egg phosphatidylcholine, in order to minimize losses (Huang, 1969). The liposome fraction containing the enzyme was eluted (buffer C) from the column in the void volume as a turbid suspension. The peak fraction (2 ml) was used for the transport experiments. P, was determined as described by Bartlett (1959).

**Kinetics**

Initial rates of NAD⁺ hydrolysis catalysed by NAD⁺ glycohydrolase reassociated into liposomes, obtained by the cholate-dialysis procedure and isolated by centrifugation in a Metrizamide gradient, were determined titrimetrically as described previously (Schuber & Travo, 1976), at pH 7.4, at 37 °C and at 10.1 (NaCl). The kinetic parameters were calculated as described by Wilkinson (1961).

**RESULTS AND DISCUSSION**

Reconstitution of detergent-solubilized proteins into artificial phospholipid vesicles is an excellent tool with which to study protein–lipid interactions and to gain information on the structural aspects of their mode of integration into membranes (Racker, 1979; Eytan, 1982; Etemadio, 1985).

**Incorporation of NAD⁺ glycohydrolase into liposomes**

In order to assess further the amphiphatic nature of calf spleen NAD⁺ glycohydrolase, we have compared the abilities of the detergent-solubilized and the water-soluble forms of the enzyme to reassociate into artificial phospholipid vesicles. Integral membrane proteins have been reconstituted in artificial lipid membranes by numerous procedures (Racker, 1979; Eytan, 1982; Etemadio, 1985), among which the cholate-dialysis technique (Kagawa & Racker, 1971) has proven successful with a variety of proteins. The degree of protein incorporation is influenced by several parameters, such as the concentration of detergent and the lipid composition of the mixture, and is dependent on the phospholipid/protein ratio (Bauman et al., 1980).

Using egg phosphatidylcholine, which has a low phase-transition temperature, we found that detergent-solubilized NAD⁺ glycohydrolase could be incorporated into vesicles by the cholate-dialysis procedure; the optimum efficiency was observed when the lipid/protein ratio was ≥ 12. A fully active enzyme preparation was thus obtained, indicating that NAD⁺ glycohydrolase retains its native conformation in a phosphatidylcholine environment. The association of the enzyme with lipid vesicles was demonstrated by flotation centrifugation in
NAD⁺ glycohydrolase reassociation into liposomes

Fig. 1. Association of calf spleen NAD⁺ glycohydrolase with [14C]phosphatidylincholine vesicles

Samples of liposome preparations (0.5 ml) obtained by the cholate-detergent procedure, as described in the Materials and methods section, were adjusted to 70% (w/v) sucrose and placed at the bottom of a 20 ml centrifuge tube. A linear sucrose gradient (50–10%, w/v) in 10 mM-potassium phosphate buffer, pH 7.4, was layered on top of the sample and was centrifuged for 30 h at 100000 g in a Beckman SW 27 rotor at 4 °C. Fractions (0.6 ml), collected from the top, were assayed for density, radioactivity (□) and for enzyme activity by the fluorimetric method (■). ΔF represents the increase in fluorescence (arbitrary units) at 410 nm (excitation of 300 nm), it monitors the hydrolysis of 1,6-etheno-NAD⁺.

(a) Vesicles prepared from egg phosphatidylincholine in the presence of detergent-solubilized NAD⁺ glycohydrolase; (b) vesicles prepared in the presence of the purified water-soluble form of the enzyme.

Sucrose density gradients; as shown in Fig. 1, a centrifugation at equilibrium allowed us to isolate a liposome population that had incorporated 75% of the detergent-solubilized form of NAD⁺ glycohydrolase (Fig. 1a); the remainder, which did not reassociate, was found at the bottom of the tube. The peak of NAD⁺ glycohydrolase activity coincided well with that of the [14C]phospholipids, at a mean density of about 1.13 g/cm³. Vesicles which were prepared similarly, but in the absence of enzyme, equilibrated at a lower density, i.e. 1.10 g/cm³ (results not shown). In contrast, the water-soluble form of NAD⁺ glycohydrolase that is produced by a limited proteolysis is unable to reassociate to the vesicles (Fig. 1b), indicating that this truncated form of the enzyme lacks the domain responsible for the anchoring of the protein to membranes. This striking difference in behaviour of the two forms of the enzyme is well correlated with their difference in interaction with hydrophobic gels (Schuber et al., 1980). Both enzyme forms also differ in their molecular masses, i.e. the detergent-solubilized NAD⁺ glycohydrolase has a molecule mass higher by about 8 kDa (Tarnus, 1985). It appears, then, that the detergent-solubilized NAD⁺ glycohydrolase is the intact form of the enzyme, which is composed of a catalytically active hydrophilic domain and a hydrophobic anchor that can be cleaved off. We do not know at present whether NAD⁺ glycohydrolase has a single membrane-spanning region, as is the case in most amphipathic proteins.

Orientation of NAD⁺ glycohydrolase incorporated into liposomes

To gain more insight into the mechanism of the insertion of detergent-solubilized NAD⁺ glycohydrolase into liposomes, we have studied the topology of reassociation. Depending on the reconstitution procedure and on the membrane-protein type, the orientation can be random or unidirectional (Eytan, 1982). The liposomes obtained by the cholate-dialysis procedure and which had incorporated the enzyme are osmotically active (Fig. 1), and these vesicles, when assayed in isoosmolar media, expressed NAD⁺ glycohydrolase activity. The kinetic parameters of this liposome-associated enzyme were determined, and the Kₘ, 79 μM, is close to the value found for the membrane-bound form of the NAD⁺ glycohydrolase, i.e. 60 μM (Travo et al., 1979). Permeabilization of the liposomes with detergents, e.g. sodium cholate (0.6% w/v) or Emulphogene (1.0%, w/v), did not increase the number of active sites accessible to substrate. Since NAD⁺ does not readily permeate phospholipid membranes, and since NAD⁺ glycohydrolase activity is unaffected by those detergents (Muller & Schuber, 1980), such observations suggest that the active site of the enzyme is located on the outer surface of the vesicles. In order to confirm this sidedness, which implies an asymmetric reassociation of NAD⁺ glycohydrolase into liposomes, we have investigated the mode of integration of the enzyme into large unilamellar vesicles obtained by detergent removal on Sephadex columns by the method of Enoch & Strittmatter (1979). This technique allows the encapsulation of solutes within the vesicles during the reconstitution. We have trapped 5(6)-carboxyfluorescein; this non-permeant fluorescent molecule is, at pH 7.4, a good marker of vesicle integrity (Weinstein et al., 1984). It exhibits a considerable self-quenching at concentrations greater than 10 mM, which is eliminated by dilution after permeabilization of the vesicles membrane. A functional NAD⁺ glycohydrolase
Table 1. Influence of liposome permeabilization on the activity of associated NAD⁺ glycohydrolase

<table>
<thead>
<tr>
<th>Emulphogene (%)</th>
<th>A vesicles (fluorescence*)</th>
<th>B vesicles (NAD⁺ glycohydrolase activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.15</td>
<td>0.47</td>
</tr>
<tr>
<td>0.001</td>
<td>0.14</td>
<td>0.47</td>
</tr>
<tr>
<td>0.002</td>
<td>0.15</td>
<td>0.47</td>
</tr>
<tr>
<td>0.005</td>
<td>0.3</td>
<td>0.47</td>
</tr>
<tr>
<td>0.01</td>
<td>0.4</td>
<td>0.48</td>
</tr>
</tbody>
</table>

* Arbitrary units.

ΔF/Δt [ΔF = fluorescence change/min at 410 nm (λ_εxcitation = 305 nm); F₀ = initial fluorescence].

was successfully reassociated into egg phosphatidylcholine liposomes by this procedure. The vesicles were separated from free 5(6)-carboxyfluorescein and unassociated enzyme respectively by the filtration step on Sephadex G-75 and by flotation centrifugation in a discontinuous Metrizamide gradient. The tightness of the vesicles was assessed by the fluorescence of trapped carboxyfluorescein which remained constant during the time of our experiments. It is noteworthy that insertion of NAD⁺ glycohydrolase into the bilayer of the liposome did not promote their leakiness, i.e. the vesicles remained impermeable and retained their internal aqueous space. As indicated in Table 1, addition of increasing concentrations of Emulphogene to the vesicles led to a doubling of fluorescence intensity, the threshold being in the 0.002–0.005 % (w/v) range. This doubling indicates that 5(6)-carboxyfluorescein was trapped to the extent of about 10 μM. This permeabilization did not result in a parallel increase of the enzyme activity associated with the vesicles, indicating that most of the liposome-associated NAD⁺ glycohydrolase is asymmetrically arranged, its active site exposed at the surface.

The sidedness of reconstituted proteins can be influenced by several factors (Eytan, 1982; Etemadio, 1985); for example, an asymmetric incorporation can, in principle, be induced by the high curvature of the bilayers and the limited inner volume of small vesicles, such as the ones obtained by the cholate-dialysis procedure (Brunner et al., 1976). Since NAD⁺ glycohydrolase was also asymmetrically reassociated into large unilamellar vesicles obtained by the Enoch & Strittmatter (1979) procedure, it seems that kinetic factors could be of importance. During detergent removal from the enzyme/phospholipid/cholate mixed micelles, by dialysis or gel filtration, phospholipid vesicles form first, the enzyme still being in solution; then, when additional detergent is eliminated, the protein inserts from the outside (Eytan, 1982), yielding a right-side-out orientation. Asymmetric reconstitution was almost always found to occur with amphipathic proteins [e.g. γ-glutamyl transpeptidase (Hughey et al., 1979), aminopeptidase (Hussain et al., 1981) and cytochrome b₅ (Enoch et al., 1979)].

Carrier capacity of liposomal NAD⁺ glycohydrolase

Besides its hydrolytic activity, NAD⁺ glycohydrolase readily catalyses transglycosidation reactions, i.e. pyridine-base exchanges, which proceed via a Ping Pong mechanism involving a reactive enzyme-ADP-ribosyl intermediate (Schuber et al., 1976). Despite the amphipathic structure of calf spleen NAD⁺ glycohydrolase, studies from our group have shown that the enzyme is much embedded into the plasma membrane, the access of its active site being sterically hindered (Muller & Schuber, 1980; Schuber et al., 1980; Muller, 1984). Moreover, in contrast with most amphipathic proteins, the hydrophilic domain of NAD⁺ glycohydrolase can only be cleaved off by proteinases when the membrane bilayer has been perturbed by detergents or by phospholipases (Schuber et al., 1980). Since the function of this ecto-enzyme remains elusive, we wanted to take advantage of the possibility of reassociating NAD⁺ glycohydrolase into artificial membranes to test whether the membrane-bound enzyme could catalyse vectorial reactions, e.g. allow the net transport of NAD⁺ molecules

Table 2. Characteristics of the liposomes used in the transport experiments

<table>
<thead>
<tr>
<th>Conditions</th>
<th>NAD⁺ glycohydrolase (nmol/min)</th>
<th>Formate dehydrogenase (nmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100 (0.2%, w/v)</td>
<td>800 (68)</td>
<td>52 (1.6)</td>
</tr>
<tr>
<td>NAD⁺ (500 μM)</td>
<td>820</td>
<td>ND</td>
</tr>
<tr>
<td>NAD⁺ (500 μM) + nicotinamide (10 mM)</td>
<td>74</td>
<td>ND</td>
</tr>
</tbody>
</table>
through phospholipid bilayers. This would imply that the high-energy enzyme–ADP-riboyl intermediate, generated during catalysis, could react in the transglycosidation reaction with acceptor nicotinamide molecules coming from either side of the membrane. Such vectorial transport systems have been reconstituted in liposomes, e.g. transport of D-mannitol phosphate by transphosphorylation catalysed by ‘mannitol Enzyme II’ (Leonard & Saier, 1983).

In order to test this possibility, we had to adopt a strategy that would allow us to measure the formation of intraliposomal NAD⁺. For that purpose we reassociated detergent-solubilized NAD⁺ glycohydrolase into large unilamellar egg phosphatidylcholine vesicles prepared by the method of Enoch & Strittmatter (1979), in the presence of formate dehydrogenase. These vesicles have a better capture efficiency than the smaller vesicles obtained by the cholate-dialysis procedure. Formate dehydrogenase, a water-soluble enzyme, was destined to be trapped along with sodium formate into the intravesicular volume during the reconstitution procedure. NAD⁺ reaching this compartment would therefore be reduced to NADH, which is detected by its absorbance at 340 nm. The vesicle population, obtained as described in the Materials and methods section, when tested in the presence of detergent was found to contain both NAD⁺ glycohydrolase and formate dehydrogenase activities. This latter activity, which was not expressed when measured in intact liposomes, was sufficient to allow the detection of intraliposomal NAD⁺ formation. A representative preparation is described in Table 2. When the intact vesicles were incubated in the presence of NAD⁺ (0.5 mM) and 10 mM-nicotinamide, the NAD⁺ glycohydrolase-catalysed hydrolysis of NAD⁺ was largely inhibited (Table 2); this is due to the transglycosidase activity of the enzyme, i.e. NAD⁺ regeneration by nicotinamide exchange.

In order to study the carrier capacity of liposomal NAD⁺ glycohydrolase, the intact vesicles were incubated in the presence of 0.5 mM-NAD⁺ and 10 mM-nicotinamide up to 50 min at 37°C. Because the acceptor molecule permeates easily through membranes, it is available in both compartments of the vesicles for the transglycosidation reaction. Within the time scale of our experiments, no absorbance increase was observed at 340 nm, indicating that no appreciable intraliposomal NAD⁺ was formed. After this time period, about 50% of the initial formate dehydrogenase activity was still measurable in the vesicles when permeabilized with detergent. These results indicate: (i) that it is feasible by the procedure of Enoch & Strittmatter (1979) to encapsulate soluble proteins in vesicles which reassociate membrane-bound proteins; (ii) that intraliposomal formate dehydrogenase was latent, i.e. extraliposomal NAD⁺ did not readily cross the bilayer; (iii) that NAD⁺ glycohydrolase incorporated into the egg phosphatidylcholine vesicles does not catalyse, at a meaningful rate, transmembrane transfer of NAD⁺ via transglycosidation.

Conclusion

The experiments described in the present study present evidence that, contrary to the water-soluble form of the enzyme, the detergent-solubilized calf spleen NAD⁺ glycohydrolase can be successfully reconstituted into artificial phospholipid vesicles. The orientation of the enzyme within the liposome is essentially right-side-out. These results are in good agreement with an amphipathic structure of the NAD⁺ glycohydrolase.

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