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PURIFICATION AND COMPARATIVE PROPERTIES OF THE GLYCOPROTEIN NICOTINAMIDE ADENINE DINUCLEOTIDE GLYCOHYDROLASE FROM RAT LIVER MICROSOMAL AND PLASMA MEMBRANES

R.P. DIAUGUSTINE, T. ABE * and P. VOYTEK **
With the technical assistance of DOLORES ECKBERG

Laboratory of Pharmacology, National Institute of Environmental Health Sciences, Research Triangle Park, N.C. 27709, and Laboratory of Medicinal Chemistry and Biology, National Cancer Institute, Bethesda, Md. 20014 (U.S.A.)

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Summary

NAD glycohydrolase, or NADase (NAD^+ glycohydrolase, EC 3.2.2.5) was solubilized with porcine pancreatic lipase from isolated fractions of microsomes and plasma membranes obtained from rat livers. The enzyme from each organelle was further purified by DEAE-cellulose chromatography, gel filtration and isoelectric focusing. The solubilized, partially purified enzymes had similar molecular weights, pH-activity profiles and K_m values. Marked charge heterogeneity was observed for the microsomal enzyme on isoelectric focusing between pH 6 and 8 with maximum activity focusing at pH 8.0. Plasma membrane NADase displayed a single peak at pH 6.7. Treatment of the partially purified microsomal or plasma membrane enzyme with neuraminidase resulted in a single peak of activity on isoelectric focusing (pH 3.5—10) with a p*I* of 9.2. Polyacrylamide gel electrophoresis of either NADase revealed a periodate-Schiff positive band which was coincident with enzyme activity. Compositional analyses of the microsomal enzyme focusing at pH 8.0 confirmed the presence of hexoses, hexosamines and sialic acid. Differences in carbohydrate composition might be important in determining the subcellular distribution of this enzyme.

* Present address: Institute of Physical and Chemical Research, Hirosawa, Wako-Shi, Saitama, 351, Japan.
** Present address: Office of Toxic Substances, Environmental Protection Agency, Washington, D.C. 20460, U.S.A.

Introduction

A number of different enzymes which have been reported to degrade nucleotides in mammalian cells are associated with membranes [1—6]. One such enzyme is nicotinamide adenine dinucleotide glycohydrolase (NAD^+ glycohydrolase, EC 3.2.2.5). This enzyme has been shown to catalyze the hydrolysis of the nicotinamide *N*-ribose linkage of NAD [7]. Some mammalian NADases also permit exchange or transglycosidation reactions of the nicotinamide moiety through an adenosine diphosphate ribose-enzyme intermediate [8,9]. Both types of reactions are inhibited by nicotinamide, a reaction product. These catalytic properties have been demonstrated for the membrane bound as well as the solubilized form of NADase [10—12].

Despite the fact that gel filtration and sedimentation velocity studies of NADase have revealed a homogeneity of this protein, marked charge heterogeneity has been observed following electrophoresis of the solubilized enzyme [13]. Since charge heterogeneity is a known characteristic for some glycoproteins [14], it was purported that mammalian NADases might contain covalently-bound sugars [13]. This finding was given support with the detection of carbohydrates in NADases isolated from certain bacteria [15] and bovine seminal fluid [16].

Earlier studies have indicated that NADase is a constituent enzyme of both endoplasmic reticulum and plasma membranes of liver cells [6]. In the present report, we have compared some of the physical, chemical and catalytic properties of this enzyme isolated from both of these organelles.

Experimental procedures

Materials. Yeast β-NAD (Grade 5), nicotinamide, *N*-acetylneuraminic acid, neutral and amino sugars, gliadin, fetuin, bee venom phospholipase, soybean trypsin inhibitor and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co. Porcine pancreatic lipase (28 or 90 units/mg, *Clostridium perfringens* neuraminidase, bovine pancreatic trypsin, egg white lysozyme, yeast alcohol dehydrogenase, *Escherichia coli* alkaline phosphatase, and rabbit muscle creatine kinase were obtained from Worthington Biochemical Corporation. Bovine serum albumin (fatty acid free) was purchased from Pentex Co. Other proteins for the calibration of gel filtration columns were obtained from the above sources and Pharmacia Fine Chemicals, Inc. Fluorescamine (Fluram) was purchased from Roche Diagnostics, Inc. Amino acid standards were obtained from Pierce Chemical Co. Other chemicals were purchased as reagent grade from local commercial suppliers.

Enzyme assay. NAD glycohydrolase activity was determined in 0.1 M phosphate buffer (pH 6.5) and 1.5 mM NAD in a final volume of 2.5 ml. The tubes were incubated at 37°C for 20 min and the reaction was terminated by the addition of 0.5 ml 25% (v/v) $HClO_4$ followed by centrifugation. The amount of residual NAD in the supernatant was measured by using the cyanide adduct method [17]. A unit of enzyme is that amount which will cleave 1 μmol NAD/h under the conditions described.

Preparation of organelles. The livers of female Fischer-344 rats (150—250 g)

were used to prepare microsomal membranes and plasma membranes. Animals were killed by cervical dislocation and all subsequent operations were carried out at approx. 4°C. For each preparation of microsomes the livers of 24 rats were removed and homogenized in 2 vols. 0.25 M sucrose using a Potter-Elvehjem homogenizer equipped with a Teflon pestle. The homogenates were centrifuged for 20 min at 10 000 × *g* in a refrigerated Sorvall RC2-B centrifuge using an SS-34 rotor. The supernatant was then centrifuged in a Beckman L5-65 ultracentrifuge at 46 000 rev./min for 90 min using a 60 Ti rotor. The supernatant was decanted and the microsomes separated from the glycogen pellet by carefully agitating the tube with the aid of a mechanical vibrator.

Plasma membranes for preparative enzyme procedures were partially purified from rat livers in a manner similar to that described by Neville [18] using steps 1—11. Four rats were used for each preparation and the washed membranes were stored as pellets at −20°C for up to one month. 8—10 such frozen preparations were used for solubilization of NADase. For assessment of NAD glycohydrolase specific activity in plasma membranes enzyme determinations were made on freshly prepared membranes isolated according to the method of Ray [19].

Solubilization. The method of Green and Bodansky [20] was used for the solubilization of NADase from microsomal and plasma membranes with some modifications. The microsomal or plasma membrane pellets were resuspended to a total volume of 40 ml with 0.2 M Tris-HCl (pH 8.0)/0.001 M $CaCl_2$. The suspension was divided among two Sorvall SS-34 rotor tubes and sonicated for 15 s with a Branson Sonifier Cell Disrupter Model 140D (Heat Systems Ultrasonics, Inc.) while the tubes were immersed in cracked ice. This was repeated three times, with approx. 3 min passing between each sonication. Pancreatic lipase (28 or 90 units/mg), free of any detectable NADase activity, was added slowly while mixing until a total of 6000 units was dissolved in the sonicated suspension with the aid of a homogenizer. The suspension was brought to 60 ml with additional buffer and then shaken at 37°C for 2.5 h in a stoppered Ehrlenmyer flask. The complete digestion mixture was then frozen overnight at −20°C. The following day, the thawed suspension was incubated again for 1.5 h and then centrifuged for 2 h at 48 000 rev./min using a Beckman 60Ti rotor. The supernatant fraction containing solubilized NADase was withdrawn and used for subsequent purification procedures.

Column chromatography. Approx. 40 ml supernatant solution was applied to a DEAE-cellulose column (2.5 × 33 cm) equilibrated with 0.005 M phosphate buffer (pH 7.4) and the enzyme was eluted with the same buffer. Fractions of 12 ml were collected and the fractions, usually numbers 7—11, containing NADase activity were pooled and concentrated to about 5 ml with an Amicon ultrafiltration cell using a PM 30 Diaflo membrane. The sample was then passed through a Sephadex G-150 column (2.5 × 84 cm) which was equilibrated with 0.1 M phosphate buffer (pH 7.4). 12-ml fractions containing NADase activity, usually numbers 16—23, were pooled and concentrated to about 5 ml.

Isoelectric focusing. A 5—20% (w/v) sucrose gradient was made in the 2.5 × 40 cm glass column of an LKB 7900 Uniphor electrophoresis system with an LKB Ultragrad gradient mixer. A coolant was passed through the column

collar which maintained the apparatus at about 5°C. The gradient contained 1% LKB Ampholine carrier ampholytes at a pH range of 3.5—10.0 or 6—8. The sample containing NADase obtained by gel filtration was added to the middle of the 220 ml gradient and focusing was initiated at 500 V and a constant current of 7 mA. The voltage was increased after 16 h to 1000 V for an additional 24 h. 5-ml fractions were collected in a refrigerated enclosure and examined directly for enzyme activity. Appropriate adjustments were made to aliquots of fractions that modified the pH of the assay system. When compositional analyses were to be made on NADase the carrier ampholytes were removed by dilution and concentration in a ultrafiltration cell until no peptides were detected in the filtrate using the fluorometric assay described below.

Treatment with neuraminidase. A 1.5 ml sample of the concentrated NADase preparation partially purified from microsomal or plasma membranes using DEAE-cellulose and Sephadex G-150 column chromatography as described above was digested with 25 units of commercial chromatographically-purified *C. perfringens* neuraminidase in a mixture of 0.001 M $CaCl_2$/ 20 mg bovine serum albumin/0.1 M acetate buffer (pH 5.6) in a total volume of 3.0 ml. The digestion was carried out in a screw-cap glass tube which was incubated at 37°C for 6 h. The neuraminidase-treated enzyme was then fractionated by isoelectric focusing as described above.

Molecular weight. Molecular weight determinations of NADase were made using the zone sedimentation sucrose density gradient method of Martin and Ames [21] and by gel filtration on a Sephadex G-150 column (0.9 × 90 cm). The column was equilibrated with 0.1 M phosphate buffer at pH 7.0—7.1 and standardized with soybean trypsin inhibitor (M_r = 21 500), pepsin (M_r = 35 000), egg white ovalbumin (M_r = 43 000), bovine serum albumin (M_r = 67 000), rabbit muscle creatine kinase (M_r = 81 000) and *E. coli* alkaline phosphatase (M_r = 89 000).

Amino acid analysis. The amino acid composition of microsomal NADase was determined essentially according to the method of Spackman [22]. The lyophilized sample (salt free) was hydrolyzed in constant boiling 6 M HCl in an evacuated sealed tube in the presence of N_2 at 110°C for 24 or 48 h. Analysis of the constituent amino acids was made on a JEOL (model JLC-6AH) amino acid analyzer. Cysteine was determined as cysteic acid following treatment of the enzyme with performic acid [23].

Analysis of carbohydrates. Total neutral sugars in the enzyme was determined by the phenol-sulfuric acid method [24], using D-galactose as the standard. For the determination of hexosamines, the glycoprotein was hydrolyzed under vacuum and in the presence of nitrogen in 3 M HCl for 3—6 h at 100°C and lyophilized to remove HCl. The hydrolysate was taken up in water and applied to a column of Dowex 50Wx 8, 100—200 mesh (H^+) as described by Spiro [25]. After washing with 4—5 column volumes of water, 2 M HCl was passed through the column. The acid eluate was lyophilized and hexosamines were determined by a modified Morgan-Elson reaction [26], using D-galactosamine as a standard. Sialic acid was estimated by the method of Warren [27] after hydrolyzing the glycoprotein in 0.05 M H_2SO_4 for 1 h at 80°C.

Disc gel electrophoresis. Purified preparations of microsomal and plasma membrane NADase were analyzed by polyacrylamide disc gel electrophoresis

using Tris-HCl/glycine buffer (pH 8.9) and 7% acrylamide. The gels were stained with either Coomassie Blue for protein or periodic acid-Schiff reagent for glycoproteins [28]. Stained disc gels were stored in 7% acetic acid in the dark at 4°C prior to photography.

Protein. For the quantitative determination of proteins the method of Lowry et al. [29] was followed using bovine serum albumin as a standard. For the microdetermination of proteins fluorescamine was employed according to the procedure of Bohlen et al. [30] using albumin as a standard. Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer (model 4-8202) equipped with a 150 W Hanovia xenon arc lamp.

Results

Purification of microsomal and plasma membrane NAD glycohydrolase. The specific and total activities associated with each step of purification of NADase from microsomal membranes are given in Table I. As was previously demonstrated [20], the enzyme could be readily solubilized with pancreatic lipase. Approximately 80% of the microsomal-bound enzyme could be solubilized using the present procedure. Addition of pancreatic trypsin or bee venom phospholipase to the digestion mixture together with lipase did no significantly improve the yield of the solubilized enzyme. NADase was also readily solubilized with lipase from partially purified plasma membrane preparations of rat liver. In three separate trials, over 90% of the enzyme originally bound to plasma membranes was detected in the supernatant fraction following centrifugation of the enzyme digest. The purification procedure for the plasma membrane was identical to that used for the microsomal enzyme (Table II). A more purified preparation of plasma membranes using the method of Ray [19] had a specific activity approximately five times greater than that of microsomes. However, this ratio was not corrected for protein non-specifically associated with membranes such a soluble proteins entrapped in the microsomal vesicles.

Microsomal and plasma membrane NADase had coincident elution profiles for both DEAE-cellulose and gel filtration column chromatography. Specific measurement of the molecular weight of partially purified microsomal NADase

TABLE I

PURIFICATION OF RAT LIVER MICROSOMAL NAD GLYCOHYDROLASE

Sample	Total protein (mg)	Specific activity (units/mg protein)	Total activity (units)
1. Lipase-digested microsome suspension	1220	1.5	1830
2. Pellet from digest	570	0.4	228
3. Soluble fraction	644	2.4	1545
4. DEAE-cellulose column	130	7.8	1014
5. Sephadex G-150 column	9.9	81.0	802
6. Isoelectric focusing			
Fraction a	0.28	218	61
Fraction b	0.21	536	113
Fraction c	0.078	1316	103

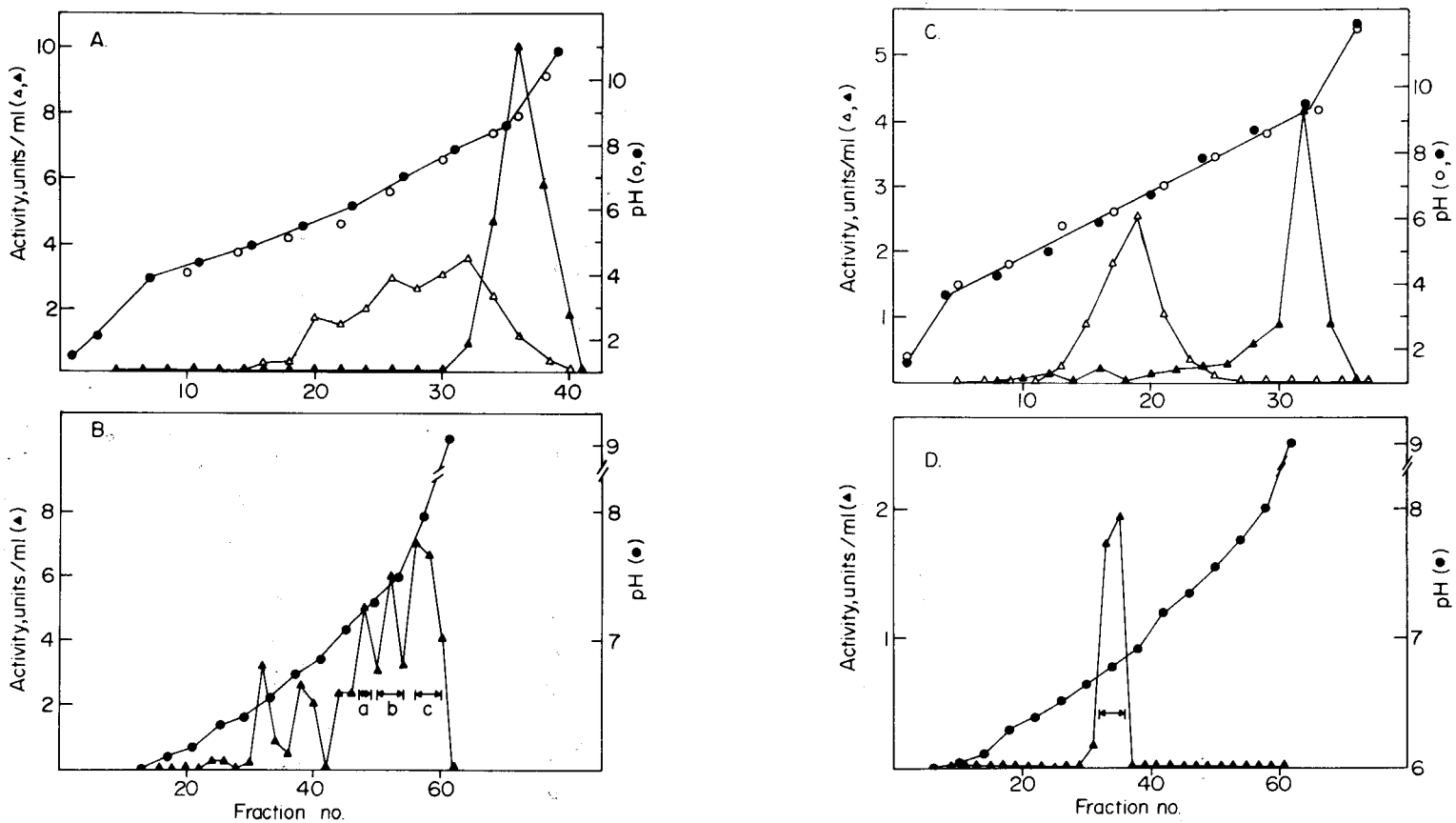

Fig. 1. Isoelectric focusing of lipase-solubilized rat microsomal and plasma membrane NAD glycohydrolase. A. Isoelectric focusing of the microsomal enzyme (△) in a pH 3.5—10.0 ampholyte gradient (○). Neuraminidase-treated NAD glycohydrolase (▲) in the same pH gradient (●) yielded a single peak with an isoelectric point of about 9.2. B. Isoelectric focusing of the microsomal enzyme (▲) in a pH 6—8 ampholyte gradient (●). C. Isoelectric focusing of the plasma membrane enzyme (△) in a pH 3.5—10.0 gradient (○). The neuraminidase-treated enzyme (▲) in the same gradient (●) yielded a single peak with an isoelectric point of about 9.2. D. Isoelectric focusing of the plasma membrane enzyme (▲) in a pH 6—8 ampholyte gradient (●). NAD glycohydrolase preparations following the Sephadex G-150 step were focused. Arrows indicate fractions that were pooled and concentrated for further studies.

TABLE II

PURIFICATION OF RAT LIVER PLASMA MEMBRANE NAD GLYCOHYDROLASE

	Total protein (mg)	Specific activity (units/mg)	Total activity (units)
1. Lipase-digested membrane suspension	227	3.9	885
2. Pellet from digest	183	0.4	73
3. Soluble fraction	40	19.9	796
4. DEAE-cellulose column	4.2	70	296
5. Sephadex G-150 column	0.9	120	108
6. Isoelectric focusing	0.05	511	24

by gel filtration or sucrose density gradient sedimentation gave values of approx. 83 000, which is in agreement with that reported by Swislocki et al. [13] for rat liver NADase. Throughout the purification procedures and prior to isoelectric focusing, fractions containing NADase from both membrane sources could be stored at −20°C for at least one month with negligible loss of enzyme activity.

Fractions from gel filtration containing NADase were pooled, concentrated and applied to an isoelectric focusing column having an ampholyte gradient from pH 3.5 to 10.0. For the microsomal enzyme, as shown in Fig. 1A, multiple peaks of activity were observed in a broad pH range beginning at about pH 5 and ending near the upper limit of the gradient. Maximum activity focused at approx. pH 8. Focusing of the microsomal enzyme in a pH 6—8 ampholyte gradient (Fig. 1B) produced finer resolution of the multiple peaks; at least five major peaks of activity were observed with this gradient. The focusing pattern of plasma membrane NADase in the pH 3.5—10.0 gradient revealed a single broad symmetrical peak with maximum activity at about pH 6.7. Following isoelectric focusing of plasma membrane NADase in the pH 6.8 gradient, maximum activity resolved at the same pH as was obtained with the gradient of pH 3.5—10.0 (Fig. 1C and D). No additional peaks could be demonstrated for the plasma membrane enzyme NADase. NADase purified from either organelle by isoelectric focusing was relatively unstable when compared to the enzyme obtained by gel filtration.

Polyacrylamide gel electrophoresis. Selected fractions from isoelectric focusing experiments were pooled, repeatedly concentrated and diluted during ultrafiltration and then analyzed by polyacrylamide gel electrophoresis. As depicted in Fig. 2A, the fraction from the focusing column (fraction c) containing the major peak of microsomal NADase activity gave a prominent band that migrated very slowly to the anode. When the gel was stained with the periodate-Schiff reagent for the detection of carbohydrates, a weak but definitely discernible band was observed in the region stained with Coomassie Blue. Simultaneous gel electrophoresis of the enzyme purified from plasma membranes revealed at least several bands; however, no bands were detected which had the same migration as that of the microsomal enzyme preparation. The most intense staining was produced in the band having the lowest degree of migration; this band also gave a weak but positive stain with the periodate-

TABLE III

AMINO ACID AND CARBOHYDRATE ANALYSES OF NAD GLYCOHYDROLASE PURIFIED FROM RAT LIVER MICROSOMES

Composition: three separate preparations of enzyme which yielded a total of approx. 0.3 mg protein obtained from fraction c by isoelectric focusing (see Table I) were pooled and used for analyses. The enzyme was hydrolyzed for 24 and 48 h. Data represent the mean of two determinations for each time period extrapolated to zero time. Values are given as molar ratios relative to methionine. Numbers are expressed as nearest integer.

	Composition
Amino acid	
Lysine	21
Histidine	6
Arginine	11
Aspartic acid	19
Threonine	13
Serine	24
Glutamic acid	24
Proline	18
Glycine	24
Alanine	21
Valine	18
Methionine	1
Isoleucine	7
Leucine	19
Tyrosine	26
Phenylalanine	8
Half-cysteine	4
Carbohydrate	
Hexoses	10.1 *
Hexosamines	1.2
Sialic acid	1.5

* Data are expressed as percent of dry weight of the enzyme.

Schiff reagent. When microsomal or plasma membrane NADase was measured in zonal sections (3 mm) of the gels, activity was only associated with the periodate-Schiff positive bands. Thus, the migration of these enzymes in the gels is in agreement with the region of the ampholyte gradient from which the respective samples were taken. For example, the microsomal enzyme which focused at the higher pH (higher p*I*) had lower anodical migration.

Compositional analyses. The preparation of significant quantities of a purified microsomal NADase enabled us to further analyze this protein. As shown in Table III, no unusual amino acid composition was observed. Since staining of the purified enzyme in acrylamide gels suggested the presence of carbohydrate, the protein was examined quantitatively for specific classes of sugars. The microsomal enzyme contained hexoses, hexosamines and sialic acid (Table III).

Charge heterogeneity. The examine whether the heterogeneity of microsomal NADase exhibited by isoelectric focusing was the result of the activity of hydrolases present during enzyme preparation, other known glycoproteins were included in the digestion mixture as possible competing substrates. When mucin, gliadin and fetuin were added together to the microsomal digestion mixture and NADase subsequently purified, no change was observed in the profile

Fig. 2. Polyacrylamide gel electrophoresis of rat microsomal (A) and plasma membrane (B) NAD glycohydrolase purified by column chromatography and isoelectric focusing using a pH 6—8 ampholyte gradient. Approx. 20 μg protein was applied to the gel in each case. The arrows indicate bands where the enzyme was detected in 3 mm zonal sections of unstained gels. These bands were weakly stained with the periodic acid-Schiff reagent. When approx. 20 μg or more of purified microsomal NADase (fraction c) was applied to polyacrylamide gels, some additional minor bands appeared in the region of the major band of stained enzyme. Analyses of the component amino acids of the major band eluted from gels and dialyzed gave essentially identical values to those reported in Table III for the protein analyzed following purification by isoelectric focusing.

of enzyme activity after isoelectric focusing (pH 6—8). Likewise, the addition of 2 mg/ml soybean trypsin inhibitor, 1 mM EDTA or 1 mM phenylmethylsulfonyl fluoride did not modify the profile of charge heterogeneity. The enzyme activity profile observed following isoelectric focusing was also independent of the duration of digestion.

Since the isoelectric point of some glycoproteins is known to be strongly influenced by the content of sialic acid [31], microsomal and plasma membrane NADase purified by DEAE-cellulose and then by Sephadex G-150 column chromatography were treated with commercial chromatographically-purified neuraminidase. The isoelectric focusing profile for the untreated

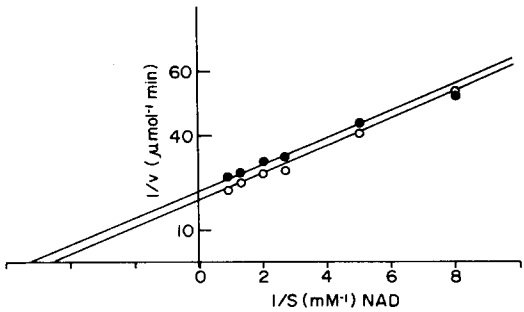

Fig. 3. Lineweaver-Burk, double-reciprocal plots for purified microsomal (•) and plasma (○) membrane NAD glycohydrolase. Each enzyme was solubilized by pancreatic lipase and purified by DEAE-cellulose and Sephadex G-150 column chromatography. Reactions were measured at 37° C.

enzyme from both sources is shown in Fig. 1A and C. Approximately 1000 units of solubilized, partially purified microsomal NADase was digested for 6 h with a recovery of 70—85% of activity. The solubilized microsomal enzyme treated with neuraminidase did not produce a heterogeneous pattern of activity when compared to the untreated preparations, but instead, yielded a single peak of activity at a p*I* of about 9.2 following isoelectric focusing. The digestion of 300 units of the partially purified plasma membrane enzyme yielded a recovery of 60—82% of enzyme activity and shifted the peak of maximal activity to a higher p*I*, essentially identical to that of the treated microsomal enzyme. Negligible activity remained at a p*I* less than 8.0. In the three separate trials for each enzyme the recoveries following digestion or subsequent isoelectric focusing were not significantly different from control experiments where neuraminidase was omitted.

Comparative kinetic properties. When pH vs. activity was studied using partially purified microsomal and plasma membrane NADase, essentially superimposable plots were obtained between pH 5 and 8. Both enzymes exhibited the same broad pH optima that had been reported in other studies of NADases [11,12,32—34]. For further comparison of the kinetic properties of the microsomal and plasma membrane glycohydrolase enzymes, double reciprocal Lineweaver-Burk plots were made using variable concentrations of NAD. As shown in Fig. 3, the microsomal and plasma membrane enzymes, partially purified by DEAE-cellulose and gel filtration chromatography, produced essentially parallel plots with similar ordinate intercepts. The K_m values for microsomal and plasma membrane NADase were $2.0 \cdot 10^{-4}$ M and $2.2 \cdot 10^{-4}$ M, respectively.

Discussion

This investigation confirms the dual localization of NADase in endoplasmic reticulum and plasma membranes obtained from homogenates of rat liver [6]. The extensive solubilization of NADase from these organelle preparations with pancreatic lipase combined with freeze-thawing provided a convenient means for obtaining these enzymes for comparative studies. Molecular weight, as determined by gel filtration, and pH optimum were identical for both enzymes.

Compositional or staining analyses of these NADases support the presence of protein-linked carbohydrates. The percentage of neutral hexoses in the purified microsomal enzyme was the same as that for the bull semen enzyme [16]. *Bacillus subtilis* and *Neurospora crassa* NADases in a previous study were also reported to contain neutral and amino sugars [15]. Although the microsomal NADase contained a lower amount of neutral sugars (as a percentage of dry weight) than either of these bacterial enzymes, the low content of amino sugars was comparable to that of the *Neurospora* enzyme. Unlike the bacterial enzymes, the hepatic NADases contain sialic acid. As judged by the marked increase in p*I* following digestion with neuraminidase, the charge heterogeneity exhibited by microsomal preparations was probably due to the variable content of sialic acid in the enzyme oligosaccharide chain. A low pK_a of 2.6 and a location as a terminal non-reducing sugar in heterosaccharides allow sialic acid to exert a major influence on the physical properties of some glycoproteins [14]. The lower p*I* and lack of noticeable charge heterogeneity exhibited by the plasma enzyme probably reflect a greater percentage of sialic acid residues over that of the microsomal enzyme. It is important to consider also that some of the peaks of enzyme activity having a relatively low p*I* in the microsomal enzyme preparation could represent contamination by plasma membranes in the microsomal fraction [35].

With the detection of sialic acid in purified microsomal NADase obtained from the peaks of major activity in isoelectric focusing experiments, it was expected that this form of the enzyme would exhibit further migration to a higher p*I* after treatment with neuraminidase. The fact that microsomal NADase and plasma membrane NADase yielded single peaks with identical mobilities following digestion with neuraminidase suggests that a major compositional variant between the enzymes from these two different cellular locations lies with the oligosaccharide chain, especially as to the relative content of sialic acid, rather than the peptide backbone. Examination of the oligosaccharide composition and structure of a purified glycoprotein has previously shown the terminal sialic acid residues to be a variable constituent [36].

It is not known from the present studies whether the catalytic function of NADase is influenced by the carbohydrates linked to the enzyme. However, the similar results obtained for microsomal and plasma membrane NADases in double reciprocal plots and pH activity curves discourage oligosaccharide composition as having any influence on catalytic function. Also, the multiple forms of NADase from rat liver [37] or calf spleen [38] yielded the same apparent K_m. Zatman et al. [8] and Yuan and Anderson [39] have shown that nicotinamide non-competitively inhibits beef spleen and bull semen NADase-catalyzed hydrolysis of NAD, respectively. In the present study, nicotinamide at 1 mM inhibited 50% of the activity of rat microsomal and plasma membrane NADases.

A dual or multi-localization of NADase in mammalian cells might be promoted by selective changes in carbohydrate composition. For proteins having a common or identical primary and subunit structures, this could be accomplished by modification of existing oligosaccharide chains. Further modification of the oligosaccharide chain of microsomal NADase by glycosyltransferases in the endoplasmic reticulum, Golgi apparatus or plasma membrane

might be required for translocation. Assuming that glycosylation of hepatic NADase is a post-translational event initiated in the endoplasmic reticulum [40], the vectorial or sequential synthesis of the oligosaccharide chain (endoplasmic reticulum → Golgi → plasma membrane or other loci) would be in agreement with a plasma enzyme variant of NADase which contains more sialic acid residues.

Some congruency can be found with the previous studies of the glycoprotein enzyme β-glucuronidase (EC 3.2.1.31) which also exhibits a multi-localization in rat liver [41]. Despite the fact that many catalytic and physical parameters were identical, the β-glucuronidase isolated from the lysosomal fraction of rat liver had a significantly lower p*I* than the enzyme isolated from the microsomal fraction [42]. The pattern of isoelectric focusing of the Golgi β-glucuronidase was similar to that of the lysosomal enzyme [43]. A specific carbohydrate sequence or heterogeneity in the carbohydrate moieties in glycoproteins could act as a determinant for the intracellular translocation or recognition of membrane-associated glycoproteins. Improved purification of the plasma membrane enzyme and finer structural analysis of these hepatic NADases should be fruitful towards understanding any specific association of protein-bound carbohydrate sequences with intracellular localization.

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