Cytosolic Location of an Endo-*N*-acetyl- β -D-glucosaminidase Activity in Rat Liver and Kidney

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Endo-*N*-acetyl- β -D-glucosaminidase activity towards an oligomannosidic type glyco-amino acid substrate was found in the soluble fraction of rat liver and kidney. No evidence for a lysosomal form of the activity was found.

Endo-*N*-acetyl- β -D-glucosaminidase (EC 3.2.1.-) catalyses the hydrolysis of the di-*N*-acetylchitobiose residue linked to asparagine in *N*-glycosidic glycopeptides. This enzyme activity has been purified from a variety of bacterial sources (Koide & Muramatsu, 1974; Tarentino & Maley, 1974; Ito *et al.*, 1975), from the fig (Ogata-Arakawa *et al.*, 1977) and from hen oviduct (Tarentino & Maley, 1976), and described in rat and pig liver (Nishigaki *et al.*, 1974). The rat liver enzyme is active against oligomannosidic-type asparagine-linked glycopeptides with optimal pH 7. It has been generally assumed (but not demonstrated) that the enzyme is lysosomal in mammalian tissues (Barrett & Heath, 1977). Evidence for the fundamental role of endo-*N*-acetyl- β -D-glucosaminidase in glycoprotein metabolism has come from the study of urinary oligosaccharides excreted by patients with lysosomal-enzyme-deficiency diseases. Urinary oligosaccharides with terminal reducing *N*-acetylglucosamine residues have been found in several different glycosidoses, leading to the hypothesis that the endo-*N*-acetyl- β -D-glucosaminidase had acted in the absence of the particular exo-glycosidase and that its action was an early step in glycoprotein catabolism (Nishigaki *et al.*, 1974; Montreuil, 1975).

We therefore considered that it was important to establish the subcellular location of endo-*N*-acetyl- β -D-glucosaminidase in rat liver and kidney to clarify its role in glycoprotein catabolism.

Materials and Methods

Materials

4-Methylumbelliferyl and *p*-nitrophenyl glycoside and phosphate substrates were obtained from Koch-Light Laboratories (Colnbrook, Bucks., U.K.). [14 C]Acetic anhydride was obtained from C.E.A. (Gif-sur-Yvette, 91190, France) and liquid scintillant OCS was from Amersham/Searle (Arlington Heights,

IL 60005, U.S.A.). 2-Acetamido-1-*N*- β -L-aspartyl-2-deoxy- β -D-glucopyranosylamine (GlcNAc-Asn) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents and buffer constituents were of analytical-reagent grade.

Endo-*N*-acetyl- β -D-glucosaminidase assay

The substrate used for the assay of endo-*N*-acetyl- β -D-glucosaminidase was glycopeptide III-A from ovalbumin (Man₅GlcNAc₄Asn) isolated as described by Tai *et al.* (1977), radioactively labelled by *N*-acetylation of the asparagine residue with [14 C]acetic anhydride and purified by high-voltage electrophoresis (Koide & Muramatsu, 1974). Assays were performed as described by Nishigaki *et al.* (1974) by incubating 0.2 nmol (5000 c.p.m.) of the substrate in 10 μ l of distilled water with 10 μ l of the appropriate buffer and 50 μ l of the enzyme fraction for 20 min–1 h at 37°C. Separation of the released GlcNAc-[14 C]Asn was achieved by using either high-voltage electrophoresis or descending paper chromatography for 16 h in the solvent system pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.) (Fischer & Nebel, 1955). The zone containing the GlcNAc-[14 C]Asn was cut out and its radioactivity counted for 10 min in liquid scintillant in an Intertechnique SL 30 liquid-scintillation counter. Specific enzyme activity was expressed as c.p.m. of GlcNAc-[14 C]Asn released/h per mg of protein.

Subcellular fractionations

Male Sprague–Dawley rats (150–200 g) were starved for 16 h overnight and then stunned by a blow on the head and killed by decapitation. Organs were removed rapidly and placed in ice-cold sucrose solution adjusted to pH 7.0 (0.25 M for the liver and 0.45 M for the kidney). Homogenates (10%, w/v) were prepared in the same sucrose solution by using one pass of a hand-rotated Potter–Elvehjem homogenizer with a loose-fitting pestle, and filtered through two

layers of muslin. Differential centrifugation of the rat liver homogenate was performed by the method of de Duve *et al.* (1955) and of rat kidney as by Shibko & Tappel (1965) to produce five subcellular fractions in each case. In addition, nuclei were purified by the method of Chauveau *et al.* (1956) and plasma membranes by the method of Ray (1970). Marker enzymes for subcellular fractions assayed were as follows: for lysosomes, acid phosphatase (EC 3.1.3.2) (Wattiaux & de Duve, 1956), *N*-acetyl- β -D-glucosaminidase (EC 3.2.1.30), β -D-galactosidase (EC 3.2.1.23) (Robinson *et al.*, 1967) and α -D-mannosidase (EC 3.2.1.24) (Opheim & Touster, 1978); for mitochondria, cytochrome *c* oxidase (EC 1.9.3.1) (Yonetani, 1967); for microsomal fraction, glucose 6-phosphatase (EC 3.1.13.9) (Ray, 1970); for the kidney soluble fraction, β -D-glucosidase (EC 3.2.1.21) (Robinson *et al.*, 1967). The plasma-membrane marker phosphodiesterase I (EC 3.1.4.1) was assayed by the method of Touster *et al.* (1970). Proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Subcellular distributions of enzyme activity are expressed in terms of the relative specific activity (de Duve *et al.*, 1955).

Results

Assay of *endo-N*-acetyl- β -D-glucosaminidase activity

Under the assay conditions used enzyme activity was linear in tissue homogenates with time and enzyme concentration up to 10% substrate utilization and was optimal at pH 7, as found by Nishigaki *et al.* (1974). One discrete peak of the reaction product (GlcNAc-[14 C]Asn) was always formed that migrated with a sample of authentic GlcNAc-[14 C]Asn on both high-voltage electrophoresis and descending paper chromatography. Paper chromatography for extended periods of time (up to 8 days) failed to separate the peak of 14 C-labelled glycopeptide after enzyme action into peaks of intermediate products formed by the action of exo-glycosidases, nor was any significant trailing of the peak noticed.

Table 1. *Subcellular distributions of marker enzymes in rat liver*

Experimental details are indicated in the text. Notation: N, nuclear fraction; M, mitochondrial-lysosomal fraction; L, lysosomal-microsomal fraction; Mic, microsomal fraction; S, soluble fraction (de Duve *et al.*, 1955). Relative specific activity is defined as specific activity in the fraction divided by specific activity in the homogenate. Results are from a representative fractionation.

Enzyme	Relative specific activity in fraction				
	N	M	L	Mic	S
<i>N</i> -Acetyl- β -D-glucosaminidase	0.6	2.6	8.0	0.9	0.1
Acid phosphatase	0.7	1.5	4.5	0.7	1.0
β -D-Galactosidase	0.8	2.1	5.6	0.8	0.3
α -D-Mannosidase	0.8	1.0	4.3	1.3	0.4
Cytochrome <i>c</i> oxidase	0.8	3.2	1.6	0.6	0
Glucose 6-phosphatase	0.8	0.7	0.9	3.2	0.8
<i>Endo-N</i> -acetyl- β -D-glucosaminidase	0.9	0.1	0.2	0.4	2.2
Protein in fraction (%)	20	11	6	20	43

Table 2. *Subcellular distributions of marker enzymes in rat kidney*

Experimental details are indicated in the text. Notation: N, nuclear; L, lysosomal-mitochondrial fraction; M, mitochondrial-microsomal fraction; Mic, microsomal fraction; S, soluble fraction (Shibko & Tappel, 1965). Relative specific activity is as defined for Table 1. Results are from a representative fractionation.

Enzyme	Relative specific activity in fraction				
	N	L	M	Mic	S
<i>N</i> -Acetyl- β -D-glucosaminidase	1.3	3.5	1.0	0.7	0.5
Acid phosphatase	1.3	3.7	0.4	0.9	0.6
Cytochrome <i>c</i> oxidase	1.4	2.3	2.3	1.3	0
Glucose 6-phosphatase	0.9	1.4	0.5	2.4	0.7
β -D-Glucosidase	0.8	0.1	0.3	0.8	2.1
<i>Endo-N</i> -acetyl- β -D-glucosaminidase	1.1	0.3	0.3	0.5	1.8
Protein in fraction (%)	12	9	22	18	39

Table 3. Recoveries of marker enzymes

Experimental details are indicated in the text. Percentage recoveries are expressed as percentages of homogenate activity recovered. Percentage activity recovered in soluble fraction is expressed in terms of the total recovered activity. Numbers in parentheses indicate numbers of experiments. N.D., Not determined.

Enzyme	Recovery (%)		Recovery in soluble fraction (%)	
	Liver (5)	Kidney (3)	Liver (5)	Kidney (3)
<i>N</i> -Acetyl- β -D-glucosaminidase	106	102	4	17
β -D-Galactosidase	87	100	19	42
α -D-Mannosidase	108	N.D.	19	N.D.
Acid phosphatase	91	96	34	30
Cytochrome <i>c</i> oxidase	101	98	0	0
Glucose 6-phosphatase	114	96	27	27
Alkaline phosphatase	N.D.	101	N.D.	11
β -D-Glucosidase	N.D.	110	N.D.	75
Endo- <i>N</i> -acetyl- β -D-glucosaminidase	85	90	67	68
Protein	102	99	35	37

Subcellular distribution of endo-*N*-acetyl- β -D-glucosaminidase

Endo-*N*-acetyl- β -D-glucosaminidase activity was recovered predominantly in the soluble fraction (S) of rat liver and kidney (Tables 1 and 2). The distributions of marker enzymes are also given in Tables 1 and 2, and indicate that, whereas very little endo-*N*-acetyl- β -D-glucosaminidase activity was recovered in the three organelle fractions mitochondrial-lysosomal (M), lysosomal-microsomal (L) or microsomal (Mic), the marker enzymes for these fractions were all distributed normally. The recovery of 96% of the (exo) *N*-acetyl- β -D-glucosaminidase activity in the particulate fractions (Table 3) indicates that lysosomes remained intact after homogenization. Measurement of latency of *N*-acetyl- β -D-glucosaminidase and acid phosphatase by the method of Price & Dance (1967) gave values of 94 and 95% respectively in the lysosomal fraction, also indicating integrity of the lysosomes. Among the enzyme markers assayed only neutral β -D-glucosidase in the kidney had a distribution similar to that of endo-*N*-acetyl- β -D-glucosaminidase, with 75% of the activity recovered in the soluble fraction (Table 3).

The proportion of endo-*N*-acetyl- β -D-glucosaminidase activity recovered in the lysosomal fraction was not increased by assaying in the presence of the detergent Triton X-100 (0.1 or 1.0%), by freezing and thawing the fraction before assay or by assaying at pH 4.5 (0.1 M-sodium phosphate/0.05 M-citric acid buffer).

Some activity (15–25% of the recovered activity) of endo-*N*-acetyl- β -D-glucosaminidase was generally recovered in the nuclear (N) fraction (Tables 1 and 2) in both liver and kidney. Further purification of liver and kidney nuclei failed to show a concomitant purification of endo-*N*-acetyl- β -D-glucosaminidase.

Plasma membranes purified more than 20-fold on the basis of the phosphodiesterase I activity contained no measurable endo-*N*-acetyl- β -D-glucosaminidase activity.

Discussion

The results obtained confirm that an endo-*N*-acetyl- β -D-glucosaminidase activity exists in rat liver and kidney, but indicate that this activity is not lysosomal but chiefly cytosolic. The activity measured was unlikely to have been due to exo-glycosidases, despite the observations by Tarentino & Maley (1976) that exo-glycosidases can contribute an apparent endo-glycosidase activity. Kinetic and chromatographic data gave no evidence for the involvement of more than one enzyme, and the pH optimum and subcellular location found suggest that exo-glycosidase activity was not involved. The concerted action of α -D-mannosidase, *N*-acetyl- β -D-glucosaminidase and β -D-mannosidase would be necessary to release GlcNAc-[¹⁴C]Asn from the substrate used according to the structure given by Tai *et al.* (1977) for glycopeptide III-A of ovalbumin. Cytosolic neutral-pH-optimal forms of the first two enzymes are known, and the cytosolic α -D-mannosidase has been shown to be active against glycopeptide substrates (Opheim & Touster, 1978), but a similar form of β -D-mannosidase has yet to be demonstrated. The possibility that action by exo-glycosidases might explain the enzyme activity is further unlikely because it failed to occur at acid pH in the lysosomal fraction.

The possible role of a cytosolic endo-*N*-acetyl- β -D-glucosaminidase could be in the catabolism of intracellular glycoproteins. The carbohydrate portion of ovalbumin glycopeptides can be extensively hydro-

lysed by purified lysosomal preparations in long incubations (LaBadie & Aronson, 1973), and there is no reason to suppose that an endo-*N*-acetyl- β -D-glucosaminidase activity is implicated. Intravenously administered asialo-glycoproteins of *N*-acetyl-lactosaminic type are accumulated rapidly in rat liver, and after a short time are localized and subsequently degraded in the lysosomes (LaBadie *et al.*, 1975). Thus a cytosolic endo-*N*-acetyl- β -D-glucosaminidase would seem to have no role in the degradation of extracellular glycoproteins. However, in cases of exo-glycosidase-deficiency diseases, the lysosomal storage diseases, in which oligosaccharides with one terminal reducing *N*-acetylglucosamine residue are excreted in the urine, an endo-*N*-acetyl- β -D-glucosaminidase would seem to be implicated (Montreuil, 1975). This activity against *N*-acetyl-lactosaminic-type glycopeptides remains to be demonstrated.

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