Rapid Papers

(Pages 261–287)

Demonstration and Cytosolic Location of an Endo-*N*-acetyl- β -D-glucosaminidase Activity towards an Asialo-*N*-acetyl-lactosaminic-Type Substrate in Rat Liver

Raymond J. PIERCE, Geneviève SPIK and Jean MONTREUIL

Laboratoire de Chimie Biologique et Laboratoire Associé au C.N.R.S. no. 217, Université des Sciences de Lille 1, B.P. no. 36, 59650-Villeneuve d'Ascq, France

(Received 24 September 1979)

An endo-*N*-acetyl- β -D-glucosaminidase activity towards an asialo-*N*-acetyl-lactosaminic-type glycoasparagine substrate was demonstrated in rat liver. This activity was optimal at pH 7.0 and was predominantly present in the soluble (cytosolic) fraction.

We have recently described (Pierce *et al.*, 1979) the presence of an endo-*N*-acetyl- β -D-glucosaminidase activity towards an oligomannosidic-type glycoasparagine derived from ovalbumin in the soluble (cytosolic) fraction of rat liver. Investigations of the oligosaccharides excreted in the urine of patients with various congenital deficiencies in lysosomal hydrolases had led to the hypothesis that an endo-*N*-acetyl- β -D-glucosaminidase had acted in the absence of the particular exoglycosidase (Nishigaki *et al.*, 1974; Montreuil, 1975). Since our previous studies had used a glycoasparagine substrate of a structural type not found in glycoproteinosis urines, we decided to investigate endo-*N*-acetyl- β -D-glucosaminidase activity in rat liver towards an *N*-acetyl-lactosaminic-type glycoasparagine isolated from human serotransferrin (Spik *et al.*, 1975). After desialylation this biantennary glycoasparagine has terminal non-reducing galactose residues, and the action of endo-*N*-acetyl- β -D-glucosaminidase would yield an oligosaccharide with a residue of *N*-acetylglucosamine in the terminal reducing position. This oligosaccharide has been described in the urine of patients with G_{M1} gangliosidosis (Wolfe *et al.*, 1974), a β -D-galactosidase-deficiency disease. We were interested to determine whether such an endo-*N*-acetyl- β -D-glucosaminidase activity normally exists in rat liver, and whether or not it is lysosomal.

Materials and Methods

Materials

4-Methylumbelliferyl glycosides, *p*-nitrophenyl glycosides and phosphate substrates were obtained from Koch-Light Laboratories (Colnbrook, Bucks., U.K.). [14 C]Acetic anhydride and NaB^3H_4 were from the Centre d'Énergie Atomique (Gif-sur-

Yvette, France). Liquid scintillant OCS was from Amersham-Searle (Arlington Heights, IL, U.S.A.). 2-Acetamido-1-*N*-(4-L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (GlcNAc-Asn) and galactose oxidase were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents and buffer constituents were of analytical-reagent grade.

Preparation of glycoasparagine substrates

The biantennary asialo-glycoasparagine of human serotransferrin was isolated by proteinase hydrolysis as described by Spik & Montreuil (1969). The substrate was radioactively labelled either by [14 C]acetylation of the asparagine residue as described by Koide & Muramatsu (1974) or by ^3H -labelling of the terminal non-reducing galactose residues by galactose oxidase treatment followed by reduction with NaB^3H_4 as described by Morell *et al.* (1966). Both radioactive products were purified by gel filtration on a column (60 cm $\times$ 2.5 cm) of Sephadex G-25 (Pharmacia France S.A., Le Chesnay, France).

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

Routine assays of endo-*N*-acetyl- β -D-glucosaminidase activity were carried out by detecting the formation of GlcNAc-[14 C]Asn liberated from the appropriate substrate. The glyco[14 C]asparagine (0.2 nmol; 10000 c.p.m.; in 20 μl of water) was incubated with 20 μl of the enzyme fraction and 20 μl of the appropriate buffer for 1 h at 37°C. Separation of the GlcNAc-[14 C]Asn formed and expression of enzyme activity was as previously described (Pierce *et al.*, 1979). The liberation of the [^3H]oligosaccharide from the [^3H]glycoasparagine was also demonstrated by using descending paper chromatography. The presence of the [^3H]oligosaccharide was

demonstrated by its co-migration with the authentic oligosaccharide after descending paper chromatography for 9 days in the solvent system pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.) (Fischer & Nebel, 1955). The authentic oligosaccharide was isolated from the urine of a patient with G_{M1} gangliosidosis and was a gift from Dr. G. Strecker. In addition, the oligosaccharide $(\text{Gal})_2(\text{GlcNAc})_2(\text{Man})_3(\text{GlcNAc})_2$ was a gift from Dr. Ph. Debeire; it was prepared from the asialo-glycoasparagine of human serotransferrin by hydrazinolysis as described by Bayard & Montreuil (1974) and reacylated with acetic anhydride (Reading *et al.*, 1978).

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 (0.25 M) adjusted to pH 7.0. Homogenates (10%, w/v) were prepared in the same sucrose solution by using one pass of a hand-operated Potter-Elvehjem homogenizer with a loose-fitting pestle and filtered through two layers of muslin. Differential fractionation of rat liver was performed by the method of de Duve *et al.* (1955) to give five subcellular fractions. Purified lysosomal extracts were prepared as described by Leighton *et al.* (1968) 34 days after an intraperitoneal injection of Triton WR-1339 (Tyloxapol; Sigma) at 85 mg/100 g rat body wt. Marker enzymes for the subcellular fractions were as previously described (Pierce *et al.*, 1979). In addition, β -D-galactosidase (EC 3.2.1.23) was assayed with the glycoasparagine substrate ^3H -labelled on the terminal non-reducing galactose residues. The substrate (0.2 nmol; 15 000 c.p.m.; in 20 μl) was incubated with 20 μl of the appropriate buffer and 20 μl of the enzyme fraction for 1 h at 37°C. Separation of the ^3H galactose liberated was achieved by using descending paper chromatography for 16 h in the Fischer-Nebel solvent system. The zone containing ^3H galactose was cut out and the radioactivity was measured by liquid-scintillation counting in an Intertechnique SC-30 scintillation counter. Specific enzyme activity was expressed as c.p.m. of ^3H galactose released/h per mg of protein.

Results

Demonstration of endo-N-acetyl- β -D-glucosaminidase activity

The liberation of $\text{GlcNAc-}^{14}\text{C}\text{Asn}$, which co-migrated with authentic $\text{GlcNAc-}^{14}\text{C}\text{Asn}$ (Pierce *et al.*, 1979), was optimal at pH 7.0 in sodium phosphate buffer (0.1 M) with a specific activity of 560 c.p.m./h per mg of protein (mean of five deter-

minations) in rat liver homogenates. This activity is 7–10-fold lower than that found for an oligomannosidic glycoasparagine substrate under the same conditions (R. J. Pierce, G. Spik & J. Montreuil, unpublished work). The ^3H oligosaccharide liberated from the ^3H glycoasparagine was separated by paper chromatography, the results of which are shown in Fig. 1. Peak I corresponded to the migration of the standard (authentic) oligosaccharide from a G_{M1} -gangliosidosis patient. Peak II was always present in the hydrolysates and co-migrated with the standard $(\text{Gal})_2(\text{GlcNAc})_2(\text{Man})_3(\text{GlcNAc})_2$, i.e. the glycoasparagine after the action of an aspartamido hydrolase cleaving the asparagine residue from the oligosaccharide chain. The presence of both peaks I and II indicates that there was the simultaneous action of endo-N-acetyl- β -D-glucosaminidase and aspartamido hydrolase on the glycoasparagine substrate, which corresponded to peak III (Fig. 1).

In parallel experiments under identical conditions the release of $\text{GlcNAc-}^{14}\text{C}\text{Asn}$ from the glyco- ^{14}C asparagine corresponded to a 1.8% hydrolysis of the substrate compared with a 1.9% production of the ^3H oligosaccharide from the ^3H glycoasparagine substrate. At the same time a 2.1% hydrolysis of the terminal non-reducing ^3H galactose residues occurred.

Fig. 1. Separation of the hydrolysis products from the ^3H glycoasparagine by paper chromatography

Products of the incubation of rat liver homogenate with the ^3H glycoasparagine substrate (for conditions see the text) were separated by descending paper chromatography in pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.) (Fischer & Nebel, 1955). Background radiation has been subtracted. The migration of the non-radioactive standards is shown on the guide strip.

Subcellular location of the activity

Endo-*N*-acetyl- β -D-glucosaminidase activity towards the *N*-acetyl-lactosaminic-type substrate was primarily located in the soluble fraction of rat liver. Fig. 2 indicates the distribution of activity in terms of the relative specific activity (de Duve *et al.*, 1955), and Table 1 shows the percentage recoveries of the activity in the various fractions. Also shown in Fig. 2 and Table 1 is the distribution of β -D-galactosidase

Fig. 2. Subcellular distribution of enzyme activities in rat liver

(a) Endo-*N*-acetyl- β -D-glucosaminidase; (b) β -D-galactosidase ($[^3\text{H}]$ glycoasparagine substrate). Activities are expressed as relative specific activity, i.e. specific activity found in the fraction/specific activity measured in the homogenate. Results from a representative fractionation are shown. Fractions are: N, nuclear; M, mitochondrial; L, lysosomal; Mic, microsomal; S, soluble (cytosolic).

activity towards the $[^3\text{H}]$ glycoasparagine substrate assayed at pH 7. As is clear, the distributions of endo-*N*-acetyl- β -D-glucosaminidase and (exo-) β -D-galactosidase are very different. β -D-Galactosidase activity in the liver at pH 7.0 is predominantly lysosomal, as shown by the high relative specific activity in the L fraction (see Fig. 2 legend), and 79% of the activity was recovered in the particulate fractions. In contrast, the endo-*N*-acetyl- β -D-glucosaminidase activity (measured by the release of GlcNAc- $[^{14}\text{C}]$ -Asn) is predominantly (59%) recovered in the soluble (S) fraction and very little enzyme activity was detected in the L fraction.

When an enriched lysosomal fraction was prepared after the administration of Triton WR-1339 no endo-*N*-acetyl- β -D-glucosaminidase activity was detectable in the tritosome fraction, although the preparation was enriched 35-fold compared with the tissue homogenate in *N*-acetyl- β -D-glucosaminidase and β -D-galactosidase activities assayed with the *p*-nitrophenyl glycoside substrates. The tritosome fraction was also enriched 25-fold in β -D-galactosidase activity at pH 7.0 towards the $[^3\text{H}]$ glycoasparagine substrate. Treatment of the tritosomes with Triton X-100 (0.5% final concentration) or sodium deoxycholate (0.5%) failed to reveal any latent endo-*N*-acetyl- β -D-glucosaminidase activity either at pH 7.0 or at pH 4.0 (0.1 M-sodium phosphate/0.05 M-citric acid buffer).

To determine whether an endo-*N*-acetyl- β -D-glucosaminidase activity was present on homogenization of the tissue but subsequently lost during differential fractionation, endo-*N*-acetyl- β -D-glucosaminidase activity was measured in a tissue homogenate (10%, w/v, in 0.25 M-sucrose adjusted to pH 7) that had been kept at 4°C for 5 h. After this time 20% of the initial activity against the *N*-acetyl-lactosaminic-type substrate at pH 7 had been lost. However, there was no evidence for any activity at acid pH in freshly prepared homogenates.

Discussion

The presence of an endo-*N*-acetyl- β -D-glucosaminidase activity in rat liver towards an asialo-*N*-acetyl-lactosaminic-type glycoasparagine substrate

Table 1. Recoveries of enzyme activities in subcellular fractions of rat liver

Experimental details are given in the text. Results are from a representative fractionation. Fractions are: N, nuclear; M, mitochondrial; L, lysosomal; Mic, microsomal; S, soluble (cytosolic). β -D-Galactosidase activity was measured with the $[^3\text{H}]$ glycoasparagine substrate.

Fraction ...	% of recovered activity					Total recovery (%)
	N	M	L	Mic	S	
Endo- <i>N</i> -acetyl- β -D-glucosaminidase	24.5	3.5	0.5	10	55.5	92
β -D-Galactosidase	9	30	27	14	21	102

has been demonstrated by both the release of GlcNAc[^{14}C]Asn and the release of the corresponding [^3H]oligosaccharide. The fact that a product comigrating with the authentic biantennary oligosaccharide is released from the [^3H]glycoasparagine substrate is evidence that the observed endo-*N*-acetyl- β -D-glucosaminidase activity does not require the prior action of exoglycosidases to reveal an unsubstituted α -(1 $\rightarrow$ 3)-linked mannose residue as is the case for endo-*N*-acetyl- β -D-glucosaminidase D isolated from *Diplococcus pneumoniae* (Ito *et al.*, 1975). The presence of another product in the reaction mixture migrating more slowly than the oligosaccharide with one *N*-acetyl-D-glucosamine residue at the reducing terminal but identically with the oligosaccharide with two terminal *N*-acetyl-D-glucosamine residues indicates that an aspartamido hydrolase was also able to act on the substrate in these conditions.

The subcellular distribution of the endo-*N*-acetyl- β -D-glucosaminidase activity is very similar to that already described towards an oligomannosidic-type substrate (Pierce *et al.*, 1979). The apparent cytosolic location of this enzyme activity and its optimal pH of 7.0 provide further evidence that exoglycosidase action was not responsible for the observed endo-*N*-acetyl- β -D-glucosaminidase activity. The predominantly lysosomal location of the β -D-galactosidase activity towards this substrate even at pH 7.0 further reinforces this view. Although some activity was undoubtedly lost during the fractionation procedure, there is no evidence that this was due to the specific loss of a lysosomal activity.

The apparent absence of any lysosomal endo-*N*-acetyl- β -D-glucosaminidase activity would seem to be counter to the hypothesis that its action is an early step in the catabolism of extracellular glycoproteins (Montreuil, 1975). As Ashwell & Morell (1971) have demonstrated, serum asialo-glycoproteins are taken up by specific receptors on the cell surface membrane before being rapidly internalized and digested in the lysosomes (LaBadie *et al.*, 1975). Thus a cytosolic endo-*N*-acetyl- β -D-glucosaminidase would seem to have a limited role in such a process. A possible role for the enzyme could be in the catabolism of intracellular glycoproteins or possibly in the control of glycoprotein synthesis.

The question arises of the possible identity of the enzyme described here with that active against oligomannosidic-type substrates (Pierce *et al.*, 1979). Preliminary evidence suggests that the two activities are due to different enzyme proteins (R. J. Pierce, G. Spik & J. Montreuil, unpublished work).

This work was supported in part by the Centre National de la Recherche Scientifique (Laboratoire Associé no. 217: Relations Structure-Fonction des Constituants Membranaires) and by the Commissariat à l'Energie Atomique.

References

- Ashwell, G. & Morell, A. G. (1971) in *Glycoproteins of Blood Cells and Plasma* (Jamieson, G. A. & Greenwalt, T. J., eds.), pp. 173–189, J. B. Lippincott, Philadelphia
- Bayard, B. & Montreuil, J. (1974) *Colloq. Int. C.N.R.S.* **221**, 209–218
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- Fischer, F. G. & Nebel, H. G. (1955) *Hoppe-Seyler's Z. Physiol. Chem.* **302**, 10–18
- Ito, S., Muramatsu, T. & Kobata, A. (1975) *Biochem. Biophys. Res. Commun.* **63**, 938–944
- Koide, N. & Muramatsu, T. (1974) *J. Biol. Chem.* **249**, 4897–4904
- LaBadie, J. H., Chapman, K. P. & Aronson, N. N., Jr. (1975) *Biochem. J.* **152**, 271–279
- Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. & de Duve, C. (1968) *J. Cell Biol.* **37**, 482–513
- Montreuil, J. (1975) *Pure Appl. Chem.* **42**, 431–477
- Morell, A. G., Van den Hamer, C. J. A., Scheinberg, I. H. & Ashwell, G. (1966) *J. Biol. Chem.* **241**, 3745–3749
- Nishigaki, M., Muramatsu, T. & Kobata, A. (1974) *Biochem. Biophys. Res. Commun.* **59**, 638–645
- Pierce, R. J., Spik, G. & Montreuil, J. (1979) *Biochem. J.* **180**, 673–676
- Reading, C. L., Penhoet, E. E. & Ballou, C. E. (1978) *J. Biol. Chem.* **253**, 5600–5612
- Spik, G. & Montreuil, J. (1969) *Bull. Soc. Chim. Biol.* **51**, 1271–1285
- Spik, G., Bayard, B., Fournet, B., Strecker, G., Bouquet, S. & Montreuil, J. (1975) *FEBS Lett.* **50**, 296–299
- Wolfe, L. S., Senior, R. G. & Ng Ying Kin, N. M. K. (1974) *J. Biol. Chem.* **249**, 1828–1838