The core-specific lysosomal α(1-6)-mannosidase activity depends on aspartamidohydrolase activity

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The substrate specificity of the core-specific rat liver lysosomal α(1-6)-mannosidase was investigated using mannosylated oligosaccharides and glycoasparagines. Hydrolysis of Man(α1-6) linkage hydrolysis was demonstrated to follow the action of endoglycosidases, namely aspartyl-*N*-acetyl-β-D-glucosaminidase and endo-*N*-acetyl-β-D-glucosaminidase. The results are discussed with respect to the nature of the carbohydrate materials stored in the tissues and excreted in the urine from patients suffering from aspartylglucosaminuria and fucosidosis.

INTRODUCTION

Lysosomal α-D-mannosidase (EC 3.2.1.24) is involved in the catabolism of asparagine-linked glycans of glycoproteins. This enzyme differs completely from other cellular mannosidases by its physicochemical and kinetic properties and its substrate specificity toward natural substrates (Winchester, 1984). It has an acidic pH optimum. Its activity is inhibited by swainsonine and Co^{2+} ions, and is stimulated by Zn^{2+} ions. Human and bovine lysosomal α-mannosidase has been resolved from various tissues into two structurally, immunologically and genetically related forms A and B by DEAE-cellulose chromatography (Caroll et al., 1972; Phillips et al., 1974a,b; Grabowski et al., 1980; Cheng et al., 1986). These two forms are deficient in patients with mannosidosis, whereas the neutral or cytosolic activity is unaffected (Caroll et al., 1972; Phillips et al., 1974b). The lysosomal α-mannosidase structural gene has been located on chromosome 19 in man (Champion and Shows, 1977). Substrate-specificity studies reveal that the lysosomal α-mannosidase is able to cleave the α(1-2)-, α(1-3)- and α(1-6)-mannosidic linkages in numerous high-mannose-type oligosaccharides (Tulsiani and Touster, 1987; Michalski et al., 1990; Al Daher et al., 1991; De Gasperi et al., 1991). However, evidence for a lysosomal α(1-6)-mannosidase, which is unaffected in genetic mannosidosis and specific for the trimannosyl core of complex glycans, has recently been described in human spleen and fibroblasts (De Gasperi et al., 1992; Daniel et al., 1992). The existence of such an enzyme, first postulated by Winchester and co-workers (Cenci di Bello et al., 1983; Winchester, 1984), may explain the origin of the major stored and excreted oligosaccharides; these lack the core α(1-6)-linked mannose residue, observed in cases of mannosidosis (Yamashita et al., 1980; Matsuura et al., 1981). In the present paper we further investigate the enzyme specificity with respect to different mannosylated glycoasparagines and oligosaccharides.

MATERIALS AND METHODS

Substrates

$Man_3GlcNAc_2Asn$ {Man(α1-3)[Man(α1-6)]Man(β1-4)GlcNAc-(β1-4)GlcNAc(β1-N)Asn} was prepared by Pronase digestion of Cohn's fraction IV (Tachibana et al., 1981) followed by digestion of the liberated glycoasparagines with a glycosidase cocktail. The latter contains sialidase, β-galactosidase and β-hexosaminidase activities and was generously provided by Professor S. Bouquelet. $Man_2GlcNAc_2Asn$[Man(α1-6)Man(α1-4)GlcNAc(β1-4)GlcNAc-(β1-N)Asn] was isolated from urine samples taken from patients suffering from aspartylglucosaminuria as previously described (Akasaki et al., 1976). The oligosaccharides $Man_3GlcNAc$ {Man(α1-3)[Man(α1-6)]Man(β1-4)GlcNAc} and $Man_2GlcNAc$ (1) [Man(α1-6)Man(β1-4)GlcNAc] were obtained by hydrolysis of the corresponding glycoasparagines with the immobilized endo-*N*-acetyl-β-D-glucosaminidase from a basidiomycete (Bouquelet et al., 1980). The oligosaccharide $Man_2GlcNAc$ (2) [Man(α1-3)Man(β1-4)GlcNAc] was isolated from a urine sample of a patient with mannosidosis (Strecker et al., 1976). The free amino group of the asparagine residue of the glycoasparagines $Man_3GlcNAc_2Asn$ and $Man_2GlcNAc_2Asn$ was acetylated with acetic anhydride as described by Koide and Muramatsu (1979).

Incubations and separation of the products

Rat liver lysosomal fractions were prepared as previously described (Michalski et al., 1990). The purity of the lysosomal fraction was checked by measuring the enrichment of the lysosomal marker enzymes, acid phosphatase and β-*N*-acetylglucosaminidase. The α-mannosidase optimum pH was established to be 5.0 with *p*-nitrophenylmannose as substrate. The absence of contamination by other subcellular mannosidases was checked by the use of specific inhibitors as previously described (Michalski et al., 1990). Incubations were performed at 37 °C in 200 mM sodium acetate, pH 5.0, buffer in the presence of 0.5 % Triton X-100 and 2.5 mM zinc acetate (Michalski et al., 1990). For comparative kinetic studies, approximately 1 mg of substrate was incubated with lysosomal extract for various periods of time. The incubations were stopped by the addition of 1 vol. of pure ethanol. After centrifugation [33 *g* (3000 rev. min), 15 min], the supernatant was desalted on a Bio-Gel P2 column (200–400 mesh, 1 cm × 50 cm) eluted with water. For analysis of the products resulting from the digestion of acetylated $Man_3GlcNAc_2Asn$ and $Man_2GlcNAc_2Asn$, after desalting and lyophilization, the products were hydrolysed by the endo-*N*-acetyl-β-D-glucasaminidase of a basidiomycete (Bouquelet et al., 1980). T.l.c. analyses were performed on precoated silica-gel 60 plates (Merck, Darmstadt, Germany). The plates were developed

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three times in butan-1-ol/acetic acid/water (2:1:1.5, by vol.) and bands were detected with the sulphuric orcinol spray reagent (0.2% orcinol in 20% H_2SO_4).

M.s. analysis

M.s. was performed on the products of the action of the lysosomal α-D-mannosidases on $Man_3GlcNAc$ and acetylated $Man_3GlcNAc_2Asn$. Substrate (2 mg) was incubated for 24 h with lysosomal extract as described above. After desalting and lyophilization, the samples were methylated by the method of Paz Parente et al. (1984). The methylated compounds were treated with 0.5 M methanol/HCl for 24 h at 80 °C, and the methyl glycosides were then analysed by g.l.c.–m.s. after peracetylation (in pyridine/acetic anhydride, 1:10; overnight at room temperature) under the following conditions: Girdel model 30 apparatus (Suresnes, France), glass capillary column (25 m × 0.3 mm) wall-coated with silicone OV 101 (helium pressure, 0.4 bar) coupled to a Riber Mag 10–10 mass spectrometer (Rueil-Malmaison, France). Chromatographic conditions were as follows: column temperature was 100–180 °C at 3 °C per min, then 180–240 °C at 6 °C per min. Mass spectra were recorded using an electron energy of 70 eV and ionizing current of 0.2 mA. Methyl glycosides were identified by comparison with standards as described by Fournet et al. (1981).

RESULTS

Kinetic study of lysosomal degradation of the glycoasparagines $Man_3GlcNAc_2Asn$ and $Man_2GlcNAc_2Asn$

$Man_3GlcNAc_2Asn$ and $Man_2GlcNAc_2Asn$ are mainly degraded by endoglycosidases, namely aspartyl-*N*-acetyl-β-D-glucosaminidase and endo-*N*-acetyl-β-D-glucosaminidase, giving the following products: $Man_3GlcNAc_2$ and $Man_3GlcNAc$ in the case of $Man_3GlcNAc_2Asn$ degradation (Figure 1a), $Man_2GlcNAc_2$ and $Man_2GlcNAc$ (1) in the case of $Man_2GlcNAc_2Asn$ degradation (Figure 1b). The activity of α-D-mannosidases is very low with these substrates. Figure 1(a) shows the formation of $Man_2GlcNAc_2Asn$ and $Man_2GlcNAc_2$, together with $Man_2GlcNAc$, which could, however, result from $Man_3GlcNAc$. It should be mentioned that $Man_2GlcNAc$ (1) and $Man_2GlcNAc$ (2) cannot be resolved by the t.l.c. system used. Figure 1(b) shows that the unique product of α-mannosidase activity is Man(β1-4)GlcNAc, which appears only after a 24 h incubation.

Kinetic study of lysosomal degradation of acetylated glycoasparagines $Man_3GlcNAc_2Asn$ and $Man_2GlcNAc_2Asn$

The free amino function of $Man_3GlcNAc_2Asn$ and $Man_2GlcNAc_2Asn$ was acetylated in order to cause a complete inhibition of aspartyl-*N*-acetyl-β-D-glucosaminidase (Tarentino et al., 1975) and consequently of endo-*N*-acetyl-β-D-glucosaminidase (Kuranda and Aronson, 1986; Brassart et al., 1987). Before t.l.c. analysis, the different samples were digested with endo-*N*-acetyl-β-D-glucosaminidase from a basidiomycete (Bouquelet et al., 1980). No degradation of acetylated $Man_2GlcNAc_2Asn$ is observed (Figure 1d), whereas hydrolysis of acetylated $Man_3GlcNAc_2Asn$ (Figure 1c) leads to the formation of a unique product, $Man_2GlcNAc_2Asn$.

Kinetic study of lysosomal degradation of oligosaccharides $Man_3GlcNAc$, $Man_2GlcNAc$ (1) and (2)

The tetrasaccharide $Man_3GlcNAc$ is mainly hydrolysed to $Man_2GlcNAc$ (Figure 2a). The lower compound Man(β1-4)GlcNAc starts to appear only after 8 h. The rates of hydrolysis of the trisaccharides $Man_2GlcNAc$ (1) and (2) are very different, although the degradation of these substrates gives the same product Man(β14)GlcNAc (Figures 2b and 2c). The oligosaccharide $Man_2GlcNAc$ (1), which has an external α(1-6)-linked mannose residue, is more resistant to hydrolysis than the oligosaccharide $Man_2GlcNAc$ (2), which has an α(1-3)-linked external mannose residue. Whereas hydrolysis of $Man_2GlcNAc$ (2) produces Man(β1-4)GlcNAc after only 1 h of incubation (Figure 2b), this compound appears only after 24 h of incubation in the case of $Man_2GlcNAc$ (1) (Figure 2c).

M.s. analysis

In a second series of experiments, the structure of the products resulting from the action of α-D-mannosidases on $Man_3GlcNAc$ and acetylated $Man_3GlcNAc_2Asn$ was determined by m.s. M.s. analysis of the $Man_3GlcNAc$ digest allows us to identify the presence of 2,3,4-tri-*O*-methyl mannoside and 2,4,6-tri-*O*-methyl mannoside. The molar ratios, established on the basis of the gas-chromatographic analysis (results not shown), indicate that these two tri-*O*-methyl mannosides occur in a ratio of 2:1. Thus lysosomal degradation of $Man_3GlcNAc$ leads to two different $Man_2GlcNAc$ isomers, the major one being Man(α1-6)Man(β1-4)GlcNAc (Figure 3a). Starting with acetylated $Man_3GlcNAc_2Asn$ as substrate, no endoglycosidase action was observed, and one product only was formed. Analysis of the

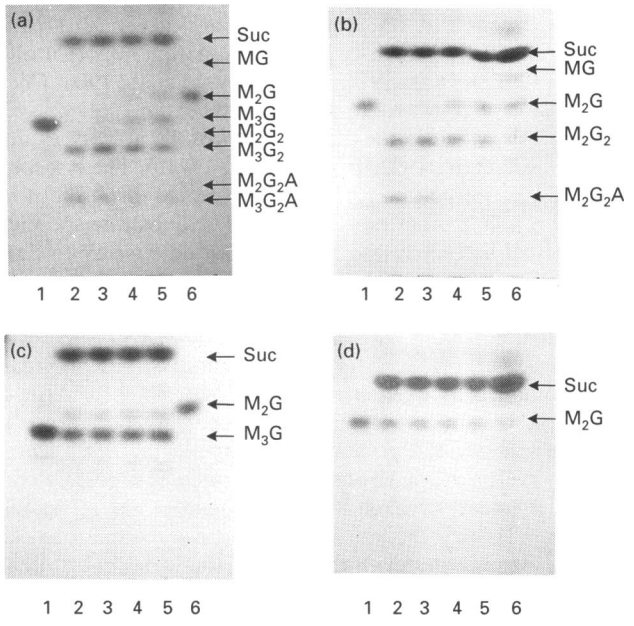

Figure 1 Kinetic study of the lysosomal degradation of the native (a, b) and acetylated (c, d) glycoasparagines, $Man_3GlcNAc_2Asn$ and $Man_2GlcNAc_2Asn$

Incubation and t.l.c. analysis were as described in the Materials and methods section. (**a**) Lysosomal degradation of $Man_3GlcNAc_2Asn$ (M_3G_2A) after 1 h (lane 2), 2 h (lane 3), 4 h (lane 4) and 8 h (lane 5). Lanes 1 and 6 are respectively $Man_3GlcNAc$ (M_3G) and $Man_2GlcNAc$ (M_2G) (2) oligosaccharide standards. (**b**) Lysosomal degradation of $Man_2GlcNAc_2Asn$ (M_2G_2A) after 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), 8 h (lane 5) and 24 h (lane 6). Lane 1 is $Man_2GlcNAc$ (2) standard. (**c**) Lysosomal degradation of acetylated $Man_3GlcNAc_2Asn$ after 1 h (lane 2), 2 h (lane 3), 4 h (lane 4) and 8 h (lane 5). Lanes 1 and 6 are respectively $Man_3GlcNAc$ and $Man_2GlcNAc$ oligosaccharide standards. (**d**) Lysosomal degradation of acetylated $Man_2GlcNAc_2Asn$ after 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), 8 h (lane 5) and 24 h (lane 6). Lane 1 is $Man_2GlcNAc$ (2) standard. Suc, sucrose; MG, ManGlcNAc; M_2G_2, $Man_2GlcNAc_2$; M_3G_2, $Man_3GlcNAc_2$.

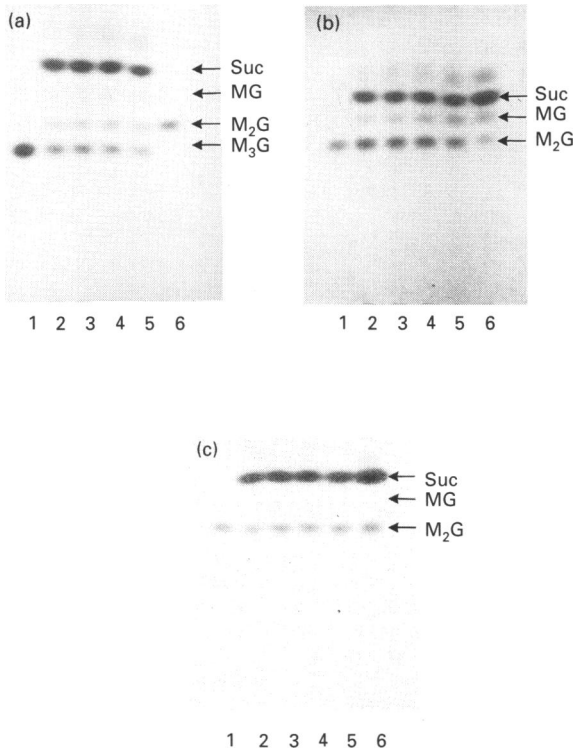

Figure 2 Kinetic study of the lysosomal degradation of oligosaccharides, $Man_3GlcNAc$ and $Man_2GlcNAc$ (1) and (2)

Incubation and t.l.c. analysis were as described in the Materials and methods section. (**a**) Lysosomal degradation of $Man_3GlcNAc$ after 1 h (lane 2), 2 h (lane 3), 4 h (lane 4) and 8 h (lane 5). Lanes 1 and 6 are respectively $Man_3GlcNAc$ and $Man_2GlcNAc$ (2) oligosaccharides standards. (**b**) and (**c**) Lysosomal degradations of Man(α1-3)Man(β1-4)GlcNAc and Man(α1-6)Man(β1-4)GlcNAc respectively after 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), 8 h (lane 5) and 24 h (lane 6). Lane 1 is $Man_2GlcNAc$ (2) standard. Abbreviations are as in Figure 1.

mass spectra shows only one tri-*O*-methyl mannoside, identified as 2,3,4-tri-*O*-methyl mannoside. The absence of the 2,4,6-tri-*O*-methyl mannoside allows us to conclude that the unique product results from α(1-3)-mannosidase activity and is Man(α1-6)-Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn (Figure 3b).

DISCUSSION

A core-specific lysosomal α(1-6)-mannosidase has recently been described in human spleen and fibroblasts (De Gasperi et al., 1992; Daniel et al., 1992). This enzyme appears to be quite different from the major lysosomal α-D-mannosidase which is able to cleave α(1-2)-, α(1,3)- and α(1-6)-linked mannose residues from high-mannose-type oligosaccharides. This α(1-6)-mannosidase can be mainly distinguished from the major one by both its inability to hydrolyse synthetic substrates and its stimulation by Co^{2+} ions (De Gasperi et al., 1992). Its activity is inhibited by swainsonine and it is unaffected in patients with genetic mannosidosis (Daniel et al., 1992). Thus it could be the residual activity originally described in the tissues of patients suffering from mannosidosis (Hultberg and Masson, 1975; Beaudet and Nichols, 1976) and it could explain the structure of the major oligosaccharide excreted in the urine of these patients (Yamashita et al., 1980; Matsuura et al., 1981). These oligosaccharides lack the core α(1-6)-linked mannose residue and could therefore arise from the action of this enzyme on the accumulated oligosaccharides that have been partially catabolized.

In the present study, we demonstrate that this core-specific α(1-6)-mannosidase is aspartyl-*N*-acetyl-β-D-glucosaminidase-dependent. This activity can only act on oligosaccharides with one GlcNAc residue at the reducing end, as also observed for the human liver enzyme (Al Daher et al., 1991). However, starting with the core $Man_3GlcNAc$ oligosaccharide as substrate, the α(1-3)-linked mannose residue is preferentially removed. Al Daher et al. (1991) also demonstrated that this enzyme cannot hydrolyse the core α(1-6)-linked mannose residue from the $Man_3GlcNAc_2$ oligosaccharide, and thus cannot act before endo-*N*-acetyl-β-D-

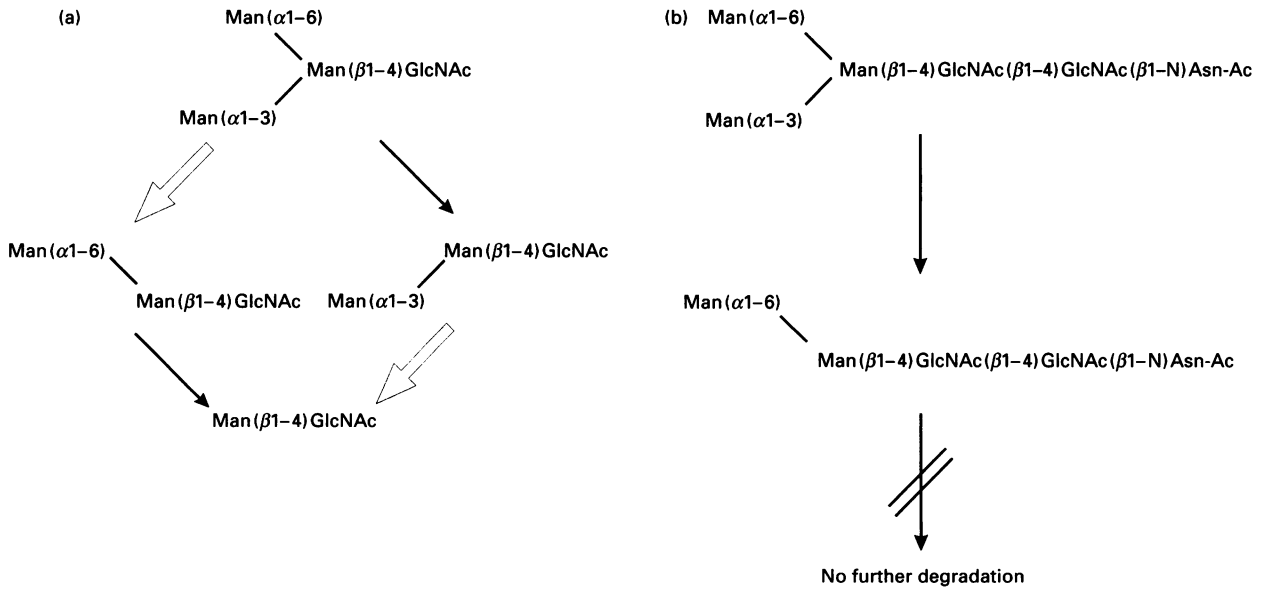

Figure 3 Schemes for hydrolysis of mannose residues from (a) tetrasaccharide $Man_3GlcNAc$ and (b) acetylated $Man_3GlcNAc_2Asn$ glycoasparagine

The relative size of the arrows reflects the intensity of the α-mannosidase activity involved.

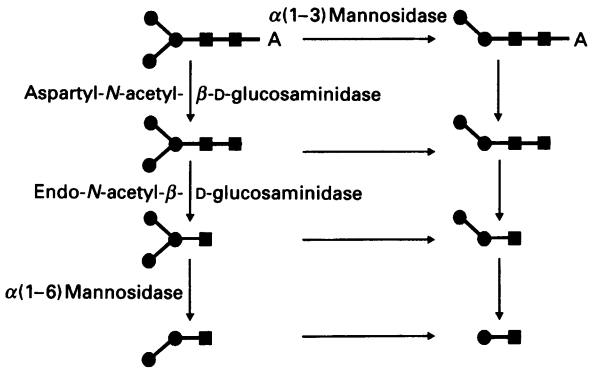

Figure 4 Schematic pathway for lysosomal degradation of the $Man_3GlcNAc_2Asn$ asparagine core of *N*-glycoproteins glycans

Explanation of the structures: ↖, Man(α1-6)Man; ●—■, Man(β1-4)GlcNAc; ↗, Man(α1-3)Man; ■—■, GlcNAc(β1-4)GlcNAc; ■—A, GlcNAc(β1-N)Asn.

glucosaminidase. Figure 4 summarizes these results and shows the degradative pathways by which the $Man_3GlcNAc_2Asn$ glycoasparagine core of *N*-glycoprotein glycans is degraded inside the lysosome. We conclude that this α(1-6)-mannosidase activity follows hydrolysis by both endoglycosidases, i.e. aspartyl-*N*-acetyl-β-D-glucosaminidase and endo-*N*-acetyl-β-D-glucosaminidase, and therefore hydrolysis by α(1-6)-fucosidase, which has been demonstrated to act after the proteases and before the endoglycosidases during catabolism of *N*-glycoproteins (Kuranda and Aronson, 1986; Brassart et al., 1987). This observation may explain the storage and excretion of the dimannosylated structure, Man(α1-6)Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]$_{0-1}$GlcNAc(β1-N)Asn, in the case of aspartylglucosaminuria and fucosidosis (Lundblad et al., 1976; Yamashita et al. 1979).

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