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The substrate-specificity of human lysosomal α-D-mannosidase in relation to genetic α-mannosidosis

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The specificity of human liver lysosomal α-mannosidase (EC 3.2.1.24) towards a series of oligosaccharide substrates derived from high-mannose, complex and hybrid asparagine-linked glycans and from the storage products in α-mannosidosis was investigated. The enzyme hydrolyses all α(1–2)-, α(1–3)- and α(1–6)-mannosidic linkages in these glycans without a requirement for added Zn^{2+}, albeit at different rates. A major finding of this study is that all the substrates are hydrolysed by non-random pathways. These pathways were established by determining the structures of intermediates in the digestion mixtures by a combination of h.p.t.l.c. and h.p.l.c. before and after acetolysis. The catabolic pathway for a particular substrate appears to be determined by its structure, raising the possibility that degradation occurs by an uninterrupted sequence of steps within one active site. The structures of the digestion intermediates are compared with the published structures of the storage products in mannosidosis and of intact asparagine-linked glycans. Most but not all of the digestion intermediates derived from high-mannose glycans have structures found in intact asparagine-linked glycans of human glycoproteins or among the storage products in the urine of patients with mannosidosis. However, the relative abundances of these structures suggest that the catabolic pathway is not the same as the processing pathway. In contrast, the intermediates formed from the digestion of oligosaccharides derived from hybrid and complex *N*-glycans are completely different from any processing intermediates and also from the oligosaccharides of composition $Man_{2-4}GlcNAc$ that account for 80–90% of the storage products in α-mannosidosis. It is postulated that the structures of these major storage products arise from the action of an exo/endo-α(1–6)-mannosidase on the partially catabolized oligomannosides that accumulate in the absence of the main lysosomal α-mannosidase.

INTRODUCTION

The lysosomal catabolism of mammalian glycoproteins containing asparagine-linked glycans is believed to occur in two stages. Firstly, the polypeptide chain is degraded by the action of endopeptidases (cathepsins), peptidases and amino- and carboxy-exopeptidases to release amino acids and glycoasparagines. The asparagine-linked glycans are then broken down in a bi-directional manner by the concerted action of exoglycosidases at the non-reducing terminal and by aspartyl *N*-acetylglucosaminidase and endohexosaminidase at the reducing terminal (Kuranda & Aronson, 1986; Brassart *et al.*, 1987; Aronson *et al.*, 1989; De Gasperi *et al.*, 1989). The exoglycosidase α-D-mannosidase (EC 3.2.1.24) catalyses the hydrolysis of α(1–2)-, α(1–3)- and α(1–6)-mannosidic linkages present in complex, hybrid and high-mannose *N*-linked glycans. In the lysosomal storage disease mannosidosis there is a genetic deficiency of α-mannosidase, and oligosaccharides with α-linked mannose residues at the non-reducing terminal accumulate in the tissues and are excreted in the urine in large amounts. Genetic mannosidosis has been described in humans (Öckerman, 1967), in cattle (Hocking *et al.*, 1972) and in cats (Burditt *et al.*, 1980). The structures of the mannose-rich oligosaccharide storage products are different in the three forms of the disease (Abraham *et al.*, 1983). In the bovine and feline forms of the disease, there are two *N*-acetylglucosamine residues (di-*N*-acetylchitobiose) at the reducing terminal because lysosomal endo-β-*N*-acetylglucosaminidase is absent in these species (Song *et al.*, 1987; De Gasperi *et al.*, 1989). In contrast, the human storage products have the composition $Man_{2-9}GlcNAc$ with a single *N*-acetylglucosamine residue at the reducing terminal because endohexosaminidase is abundant in man. The structures of the storage products in human mannosidosis are all consistent with the incomplete catabolism of asparagine-linked glycans found in glycoproteins (Norden *et al.*, 1974; Yamashita *et al.*, 1980; Matsuura *et al.*, 1981; Egge *et al.*, 1982). However, the major component, Manα(1–3)Manβ(1–4)GlcNAc, presumably derived from the digestion of complex glycans, lacks the expected mannose residue linked α(1–6) to the β-linked core mannose residue. Several other major storage products also lack this α(1–6)-linked residue. It has been suggested that a minor α(1–6)-mannosidase, which is unaffected in mannosidosis, accounts for these structures (Cenci di Bello *et al.*, 1983). To understand further the lysosomal catabolism of human glycoproteins, the digestion by human liver α-mannosidase of natural oligosaccharide substrates derived from complex, hybrid and high-mannose *N*-linked glycans and of mannosidosis storage products has been investigated. The substrate-specificities of bovine and feline lysosomal α-mannosidases have been investigated in a parallel study (De Gasperi *et al.*, 1991).

MATERIALS AND METHODS

Preparation of human liver lysosomal α-mannosidase

Liver lysosomal α-mannosidase was prepared by following the procedure of Phillips *et al.* (1976) but omitting the heat denaturation step. The preparation was assayed with the synthetic substrate 4-methylumbelliferyl α-D-mannopyranoside in phosphate/citrate buffer, pH 4.0 (Phillips *et al.*, 1974). One

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unit of enzyme activity was defined as the amount of enzyme hydrolysing 1 μmol of substrate/min at 37 °C. Human liver was homogenized in 10 mM-sodium phosphate buffer, pH 6.0. The 50% (w/v) homogenate was centrifuged at 30000 *g* in an MSE Prepspin centrifuge for 30 min at 4 °C. The clear supernatant was filtered through glass-wool, and the material precipitated between 35% and 60% saturation with $(NH_4)_2SO_4$ was collected by centrifugation. The pellet was resuspended in 10 mM-sodium phosphate buffer, pH 6.8, containing 0.1 mM-$CaCl_2$, 0.1 mM-$MnCl_2$ and 0.1 mM-$MgCl_2$ (low-salt concanavalin A buffer) before chromatography on concanavalin A–Sepharose. The acidic α-D-mannosidase activity, which bound to the affinity matrix, was eluted with the same buffer containing 0.5 M-NaCl and 0.5 M-methyl α-mannoside. This material, after dialysis, was used in all the specificity studies. The specific activities of the preparations were in the range 0.5–1.8 munits/mg of protein. Other glycosidase activities were assayed with the appropriate 4-methylumbelliferyl glycoside or *p*-nitrophenyl glycoside at the appropriate pH optimum as described previously (Phillips *et al.*, 1976). Protein was determined by using Coomassie Blue (Bradford, 1976), with BSA as the standard.

Isolation and purification of substrates

The structures, sources and abbreviations for the oligosaccharides used as substrates are shown in Tables 1 and 2. Digestions with endohexosaminidases D and H were performed as described previously (Warren *et al.*, 1988*a*,*b*), and the products were purified by chromatography on Bio-Gel P-4 (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The purity of all the substrates was checked by h.p.l.c. and h.p.t.l.c. The mixture of oligosaccharides of composition $Man_{5-8}GlcNAc$ (**5a**, **6a**, **7a/b** and **8a**) was obtained from Genzyme Corp. (Cambridge, MA, U.S.A.), and the oligosaccharide of composition $Man_9GlcNAc$ (**9**) was generously provided by Dr. S. Hirani (Genzyme Corp.). The structures of these compounds have been defined by h.p.l.c. and n.m.r. spectroscopy. After reduction $Man_8GlcNAc$ (**8a**) was isolated from the mixture by rechromatography on Bio-Gel P-4 and its homogeneity was checked by h.p.l.c.

Radiolabelling of oligosaccharides

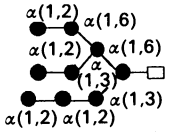
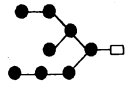
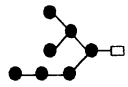
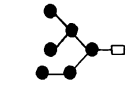
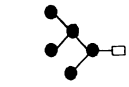
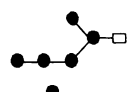
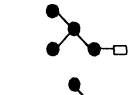
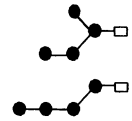
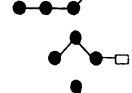
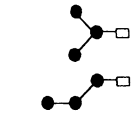
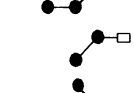
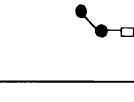
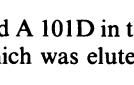
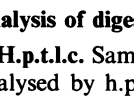
Purified oligosaccharides were radiolabelled with NaB^3H_4 by using a modification of the procedure of Mellis & Baenziger (1981). Oligosaccharides were dissolved in 20 μl of 200 mM-sodium borate buffer, pH 10.5. NaB^3H_4 (600 mCi/mmol) (New England Nuclear, Stevenage, Herts., U.K.) was added to a final concentration of 20 mM and the solution was incubated for 3 h at 30 °C. Reduction was stopped by the addition of 150 μl of 5% (v/v) acetic acid and the reaction mixture was dried down under a stream of N_2 at 40 °C, followed by two further evaporations from 250 μl of methanol. Samples were then resuspended in distilled water and applied to the Bio-Gel P-4 (200–400 mesh) column (100 cm × 2.6 cm diam.) in distilled water at a flow rate of 4 ml/h. Fractions (1 ml) were collected, and the radioactivity was determined by liquid-scintillation counting and the oligosaccharides were analysed by h.p.t.l.c. and h.p.l.c.

Incubation conditions

Routinely 15–20 nmol of substrate was incubated with 0.1–0.2 unit of α-mannosidase activity in 100 μl of phosphate/citrate buffer, pH 4.0. At different time intervals samples were removed for analysis by h.p.t.l.c. and h.p.l.c. The reaction was stopped by adding an equal volume of ethanol (95%) and boiling the sample for 3 min. The samples were then centrifuged at 8000 *g* for 3 min to remove the protein, and the neutral oligosaccharides were separated by passing the supernatant through a Duolite mixed-bed resin (MB 6113, a mixture of Duolite C225 in the H^+ form and A 101D in the OH^- form, with the inclusion of an indicator), which was eluted with deionized water.

Table 1. Structures, abbreviations, retention times and origins of the oligosaccharide substrates and standards

The linkages in the schematic representation of the structures are shown for compound (**9**), $Man_9GlcNAc_1$ [●—□ = Man-β(1–4)GlcNAc]. The retention times are representative values, corresponding to the h.p.l.c. profile shown in Fig. 1. The two isomers of $Man_7GlcNAc_1$, (**7a**) and (**7b**), with a single α1–2-linked mannose on the top and bottom branches, were not resolved by h.p.l.c. Key to origin: (i) commercial source (Genzyme Corp.); (ii) obtained by treatment with endohexosaminidase H of compounds described in Warren *et al.* (1988*a*); (iii) isolated as described in Warren *et al.* (1988*b*) and for (**3a**) after treatment with endohexosaminidase D; (iv) isolated as reported in Warren *et al.* (1983); (v) prepared by treatment of compound (**2'b**) with endoglucosaminidase isolated from human tissues (De Gasperi *et al.*, 1989).

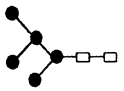
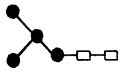
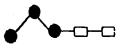
Substrate	Abbreviation and no.	Retention time (min)	Origin
	$Man_9GlcNAc_1$ (**9**)	60.55	(i)
	$Man_8GlcNAc_1$ (**8a**)	58.92	(i)
	$Man_7GlcNAc_1$ (**7a/b**)	56.23	(i)
	$Man_6GlcNAc_1$ (**6a**)	53.43	(i)
	$Man_5GlcNAc_1$ (**5a**)	50.14	(ii)
	$Man_5GlcNAc_1$ (**5b**)	48.17	(iii)
	$Man_4GlcNAc_1$ (**4a**)	45.08	(ii)
	$Man_4GlcNAc_1$ (**4b**)	42.92	(iii)
	$Man_4GlcNAc_1$ (**4c**)	42.10	(iv)
	$Man_3GlcNAc_1$ (**3b**)	36.97	(ii)
	$Man_3GlcNAc_1$ (**3a**)	36.97	(iii)
	$Man_3GlcNAc_1$ (**3c**)	35.48	(iv)
	$Man_2GlcNAc_1$ (**2a**)	22.90	(iv)
	$Man_2GlcNAc_1$ (**2b**)	22.90	(v)

Analysis of digestion products and substrates

H.p.t.l.c. Samples of the digestion mixtures or substrates were analysed by h.p.t.l.c. in pre-coated silica gel 60 plates (Merck, Darmstadt, Germany). The plates were developed twice in propan-1-ol/water (8:3, v/v). Samples and authentic standards were run on the same plate to enable direct comparison. After drying, the plates were sprayed with En^{3}Hance (New England

Table 2. Structures, abbreviations and origins of the oligosaccharide substrates and standards with two *N*-acetylglucosamine residues at the reducing terminal

Oligosaccharides with chitobiose at the reducing terminal are denoted by adding a prime to the number of the corresponding substrate with a single *N*-acetylglucosamine residue. The linkages in the schematic representations are the same as those in the corresponding structures in Table 1. Key to origin: (ii) obtained as described by Warren *et al.* (1988*a*); (iii) obtained as described by Warren *et al.* (1988*b*).

Substrate	Abbreviation and no.	Origin
	$Man_5GlcNAc_2$ (5′a)	(ii)
	$Man_4GlcNAc_2$ (4′a)	(ii)
	$Man_3GlcNAc_2$ (3′a)	(iii)
	$Man_3GlcNAc_2$ (3′b)	(ii)
	$Man_2GlcNAc_2$ (2′b)	(ii)

Nuclear) and exposed overnight against Kodak X-omat XAR-5 film at −70 °C. Individual oligosaccharides were isolated for further analysis by only spraying and exposing strips on the edges of the plate to identify the components. Material was collected from the corresponding unsprayed zone on the plate. After washing twice with water, the samples were desalted. For non-radiolabelled substrates, oligosaccharides were detected with orcinol (Cenci di Bello *et al.*, 1983).

H.p.l.c. Samples of the substrates or of the digestion mixtures of the radiolabelled substrates obtained at different time intervals were placed in stoppered silanized glass tubes. Then 12.5 vol. of acetic anhydride and 12.5 vol. of anhydrous pyridine were added. The samples were mixed very well and incubated at 60 °C for 16 h. After drying under N_2, they were redissolved and dried a further two times by evaporation of 1 ml of toluene. The final residue was resuspended in 100 μl of acetonitrile/water (7:13, v/v). The acetylated samples were analysed by h.p.l.c. (Hall & Patrick, 1989) with the use of a Spectra Physics 8700 liquid-chromatography pump, a Reeve analytical splitter mixer, a radioactivity monitor fitted with a 1 ml Teflon flow cell and a Spectrophysics 4270 integrator. The h.p.l.c. column was 15 cm × 4.6 mm Spherisorb ODS 2 (3 μm particle size) (Jones Chromatography, Llanbradach, Mid-Glamorgan, U.K.). A guard column (5 cm × 2.3 mm) packed with CO:PELL ODS (Whatman, Maidstone, Kent, U.K.) was fitted to the analytical column. Different isocratic solvent systems of acetonitrile and water were used for separation of mixtures of low-M_r and high-M_r oligosaccharides respectively. A gradient solvent system running from 33 % to 59 % (v/v) acetonitrile was used to analyse digestion mixtures. To separate derivatives of individual oligosaccharides by h.p.l.c. for analysis by acetolysis, a duplicate sample was applied to the column. The eluate was not passed through the radioactivity monitor but collected in a fraction collector. Fractions corresponding to peaks (i.e. retention times) were collected and re-analysed by h.p.l.c. before acetolysis.

Acetolysis. Samples for acetolysis were pre-acetylated as above and dried down in silanized tubes before addition of 100 μl of the acetolysis mixture, consisting of 1.1 ml of acetic anhyride, 1 ml of acetic acid and 100 μl of conc. H_2SO_4. After thorough mixing, the reaction mixture was incubated at 37 °C for 4 h and/or 16 h. The reaction was stopped with 1 ml of water, followed by 2 ml of chloroform. The lower organic layer was evaporated to dryness after two washes with water. The residue was redissolved in 100 μl of a mixture of acetonitrile/water (7:13, v/v) for analysis by h.p.l.c.

RESULTS

Lysosomal α-mannosidase

Lysosomal α-mannosidase was partially purified from human liver, the activity being monitored with the synthetic substrate 4-methylumbelliferyl α-D-mannopyranoside. The evidence that the α-mannosidase activity obtained by this procedure was due exclusively to the lysosomal form of the enzyme and not to the forms associated with the cytosol, endoplasmic reticulum or Golgi apparatus (Phillips *et al.*, 1974; Hirani & Winchester, 1979) can be summarized as follows. The integral membrane α-mannosidases of the endoplasmic reticulum and Golgi apparatus are not extracted with the aqueous solvent used in the first step of the purification procedure. If small amounts of any of these activities were extracted because of the use of frozen tissue, they would be removed by the subsequent steps in the procedure, which were designed to eliminate such contaminants and the soluble cytosolic α-mannosidase. Most of the cytosolic α-mannosidase was precipitated by $(NH_4)_2SO_4$ (0–35 % saturation), but any escaping precipitation was separated from the lysosomal α-mannosidase by affinity chromatography on concanavalin A–Sepharose, to which it does not bind (Phillips *et al.*, 1976; Hirani & Winchester, 1979). Finally, to ensure that any traces of contaminating non-lysosomal α-mannosidases do not contribute to the digestions of the natural substrates, these were performed at pH 4, at which pH the non-lysosomal enzymes have negligible activity. The effects of Co^{2+} (inhibition), Zn^{2+} (activation), swainsonine (inhibition at 10 μM) and deoxymannojirimycin (non-inhibition at 0.1 mM) on the partially purified α-mannosidase towards the synthetic substrate were all consistent with it being lysosomal α-mannosidase. Furthermore, no digestion of the natural substrates occurred in the presence of swainsonine (100 μM). Some β-D-mannosidase was present in the preparation. Although this could be removed completely by heat denaturation, this treatment was found to decrease the α-mannosidase activity towards natural but not synthetic substrates. However, human β-mannosidase did not hydrolyse mannose β(1–4)-*N*-acetylglucosaminitol and therefore did not interfere with the digestions. For all these reasons, the α-mannosidase activity employed in the digestions of natural substrates is considered to be due exclusively to the lysosomal form of the enzyme under the conditions used.

Substrates

All of the radiolabelled oligosaccharide substrates and standards were analysed by h.p.t.l.c., h.p.l.c. and acetolysis to check their purity and to establish their chromatographic properties. This information was essential for the identification of intermediates in the enzymic digestion mixtures and the elucidation of the catabolic pathways. A representative analysis of a mixture of oligosaccharides by h.p.l.c. is shown in Fig. 1. In preliminary experiments unlabelled substrates were digested and analysed by h.p.t.l.c. before and after reduction. The reduction of the substrates did not affect the pathway of catabolism. Therefore radiolabelled substrates were used because of the greater sen-

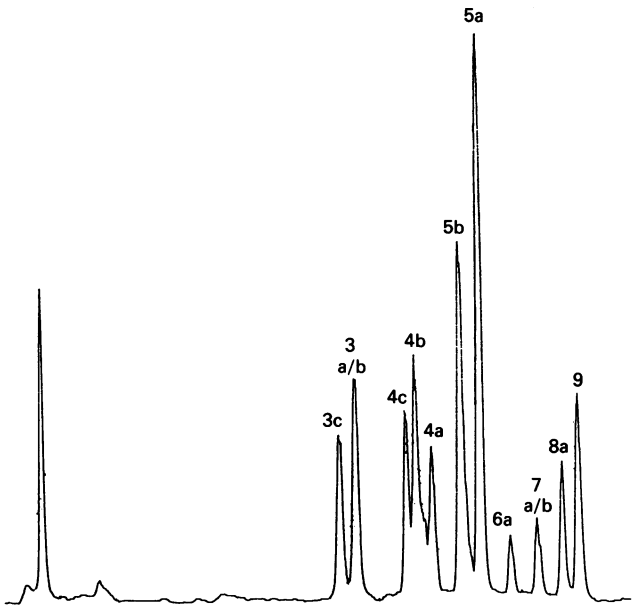
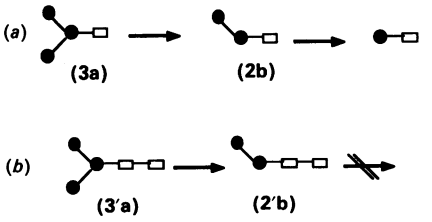


Fig. 1. H.p.l.c. of reduced oligosaccharide substrates and standards

The number of each peak corresponds to the structure of the oligosaccharide in Table 1. The separation of these different isomers was achieved by initially running the isocratic system aq. 33% (v/v) acetonitrile for 17 min. Then a gradient system running from 33% to 59% (v/v) acetonitrile was developed over 39 min. This system was used for the analysis of all the subsequent time courses.



Scheme 1.

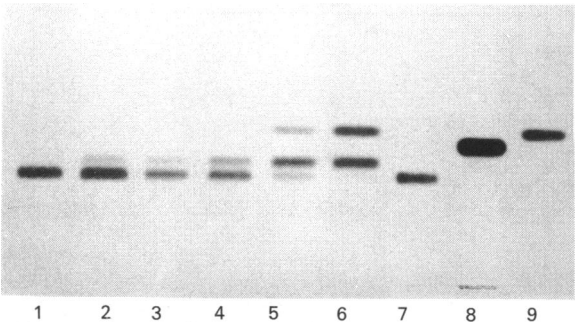


Fig. 2. Analysis by h.p.t.l.c. of the digestion of $Man_3GlcNAc_1$ (3a) by the human lysosomal α-mannosidase

Samples were removed from the digestion mixture at different time intervals and analysed by h.p.t.l.c. Lane 1, 0 h; lane 2, 2 h; lane 3, 4 h; lane 4, 6 h; lane 5, 24 h; lane 6, 48 h; lane 7, undigested substrate; lane 8, Manα(1–3)Manβ(1–4)GlcNAc (**2a**); lane 9, Manβ(1–4)GlcNAc.

sitivity and resolution of h.p.l.c. The suffix -ol is omitted from the structures of the oligosaccharide substrates for clarity but it is implied throughout unless specifically stated otherwise.

Digestion of natural substrates

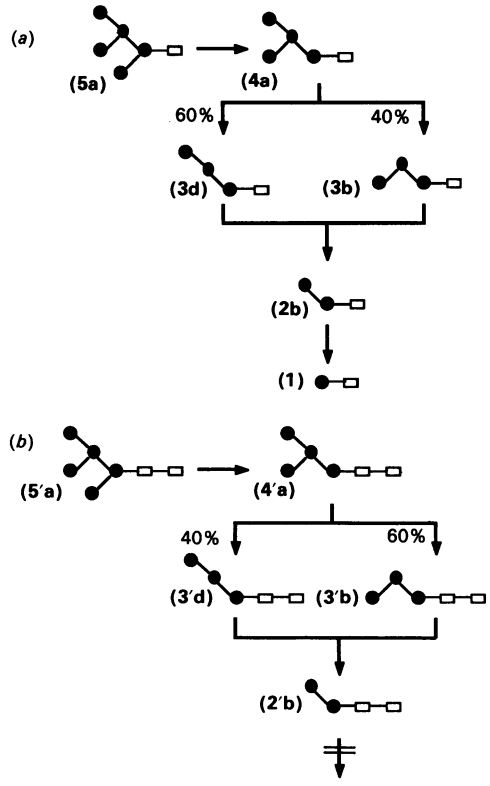
Substrates derived from complex glycans. The first substrate presented to α-mannosidase by the bi-directional digestion of complex *N*-glycans in human lysosomes would be Manα-(1–3)[Manα(1–6)]Manβ(1–4)GlcNAc (**3a**). This compound is digested *in vitro* by human lysosomal α-mannosidase in a non-random manner (Scheme 1*a*). Analysis of the digestion mixture by h.p.t.l.c. (Fig. 2) showed that the tetrasaccharide (**3a**) was hydrolysed predominantly to a unique trisaccharide. This component had a different mobility on h.p.t.l.c. from the authentic standard of Manα(1–3)Manβ(1–4)GlcNAc (**2a**) but the same mobility as Manα(1–6)Manβ(1–4)GlcNAc (**2b**). The two authentic components were not resolved on h.p.l.c., but co-injection of a digestion mixture with the two standards gave a single peak for $Man_2GlcNAc$, confirming the identity of the trisaccharide. Thus lysosomal α-mannosidase preferentially removes the core α(1–3)-linked mannose residue followed more slowly by the α(1–6)-linked mannose residue.

The α(1–3)-linked mannose residue was also removed preferentially from the corresponding substrate containing two *N*-acetylglucosamine residues (**3'a**) at the reducing terminal (Scheme 1*b*). However, in this case the α(1–6)-linked mannose residue was completely resistant to hydrolysis.

Substrates derived from hybrid glycans. An oligosaccharide containing five mannose residues and a single *N*-acetylglucosamine residue (**5a**) would be the first substrate to be presented to human lysosomal α-mannosidase from the breakdown of hybrid glycans. Analysis by h.p.l.c. of the digestion of this substrate by the human enzyme *in vitro* showed that the first step was the removal of the core α(1–3)-linked mannose residue to yield a unique pentasaccharide (**4a**) (Scheme 2*a*). The structure of this intermediate was determined by co-migration of an authentic sample in h.p.l.c. and by acetolysis (Fig. 3). Thus the core α(1–3)-mannosidic linkage is equally susceptible to hydrolysis when the core α(1–6)-mannose residue is substituted (Table 3). Subsequently a single trisaccharide (**2b**) is formed by two alternative pathways, in which one of the peripheral mannose residues is removed first, followed by the other, with the α(1–3)-linkage being more readily hydrolysed. Again the structures of the two tetrasaccharides (**3b** and **3d**) were confirmed by chromatography of isolated intermediates and authentic standards before and after acetolysis. The trisaccharide (**2b**), which was the same as that obtained in the digestion of the core of the complex glycans, was broken down only very slowly as observed previously.

The analogous substrate with di-*N*-acetylchitobiose at the reducing terminal (**5'a**) was hydrolysed by lysosomal α-mannosidase by the same route as the substrate with a single *N*-acetylglucosamine residue (Scheme 2*b*) as far as the tetrasaccharide (**2'b**), which was again resistant to hydrolysis.

High-mannose glycans. The digestion of $Man_9GlcNAc$ (**9**) by lysosomal α-mannosidase also follows a discrete pathway (Scheme 3). A specific peripheral α(1–2)-linked mannose residue is removed to generate a unique $Man_8GlcNAc$ (**8b**), which is different from the $Man_8GlcNAc$ (**8a**) available as a substrate. This unique intermediate was subsequently hydrolysed to a unique $Man_7GlcNAc$ (**7c**) and $Man_6GlcNAc$ (**6b**) (Fig. 4*a*). The structures of these intermediates were deduced by comparison with the retention times on h.p.l.c. of standards and by acetolysis of isolated intermediates (Fig. 4*b*). They were distinct from the standards (**7a/b**) and (**6a**). The $Man_6GlcNAc$ (**6b**) was hydrolysed to a mixture of three products ($Man_5GlcNAc$) (Fig. 4*a*). The predominant component (**5c**), about two-thirds, was shown by acetolysis to lack the core α(1–3)-linked mannose residue, empha-



Scheme 2.

sizing the susceptibility of this linkage to hydrolysis by lysosomal α-mannosidase. The retention time of one of the two minor $Man_5GlcNAc$ components suggested that it was compound (**5a**), and this was confirmed by acetolysis of the isolated intermediate. By deduction the structure of the remaining $Man_5GlcNAc$ had to be (**5d**) (Scheme 3). Only two $Man_4GlcNAc$ components were detected. They arose by removal of the core α(1–3)-linked mannose residue from the two minor $Man_5GlcNAc$ compounds (**5a**) and (**5d**) and by the removal of the remaining α(1–2)- or α(1–6)-linked residue from the major component (**5c**). Intermediates (**5a**) and (**5d**) can only give rise to intermediates (**4a**) and (**4d**) respectively. Therefore it can be deduced that the major intermediate (**5c**) is hydrolysed to (**4a**) and (**4d**) in the ratio of approx. 2:1. The final steps in the degradation pathway for $Man_9GlcNAc$ were similar to those observed for $Man_3GlcNAc$ (**3a**) and $Man_5GlcNAc$ (**5a**), except that the straight-chain $Man_3GlcNAc$ (**3d**) was not observed as a product.

The pathway for the lysosomal breakdown of the $Man_8GlcNAc$ substrate (**8a**) was distinct from that for the $Man_8GlcNAc$ (**8b**) derived from $Man_9GlcNAc$ (**9**) (Scheme 4). The structures of the unique isomers of composition $Man_7GlcNAc$ (**7a**), $Man_6glcNAc$ (**6a**) and $Man_5GlcNAc$ (**5a**) produced were established by retention times and acetolysis of isolated intermediates (Fig. 5*a*). The $Man_5GlcNAc$ intermediate had the same structure as the $Man_5GlcNAc$ substrate (**5a**) already investigated, and the final stages of digestion were the same as for that compound. Oligosaccharide (**5a**) is only a minor intermediate in the catabolic pathway for $Man_9GlcNAc$.

The elucidation of this pathway was aided by the use of the mixture of oligosaccharides of known structure containing five to eight mannose residues (**5a**, **6a**, **7a/b** and **8a**). Digestion of this mixture of oligosaccharides, which contains all the intermediates $Man_{5-8}GlcNAc$ in the pathway for the digestion of $Man_8GlcNAc$ (**8a**), did not generate any new or unknown intermediates (Fig.

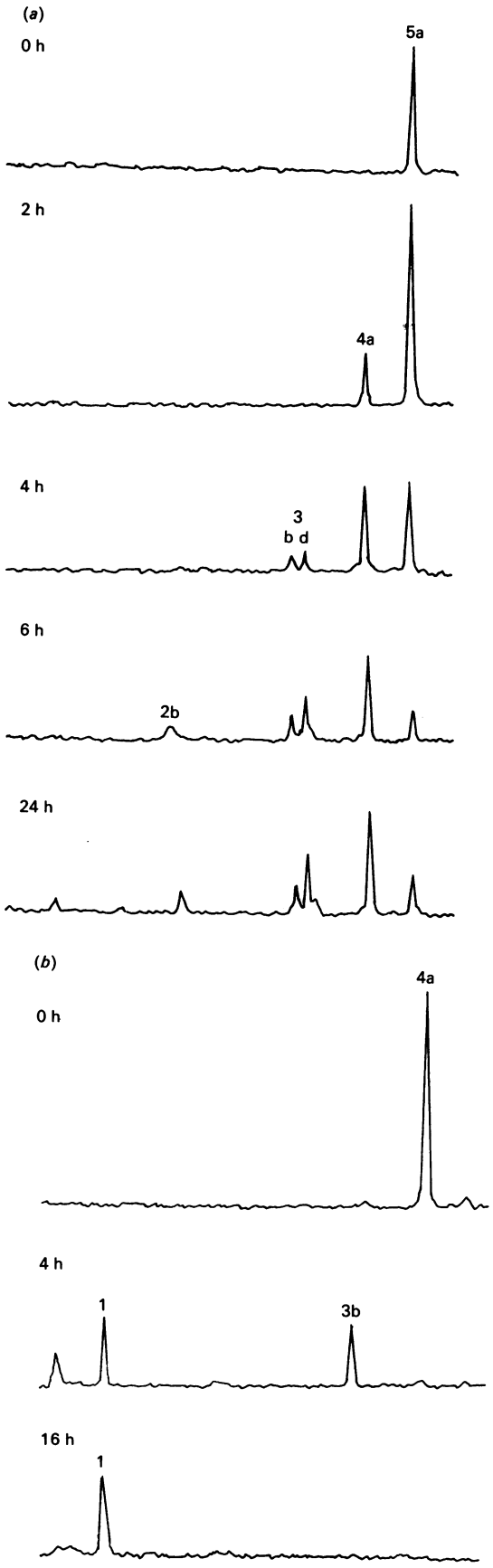


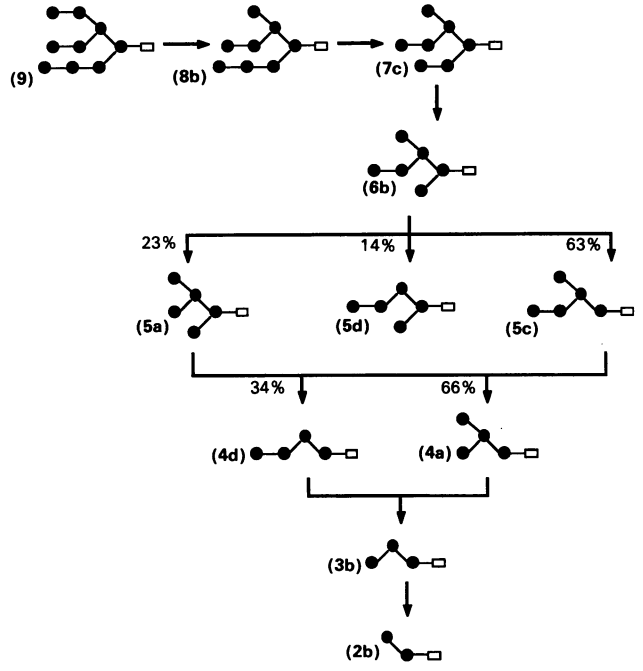
Fig. 3. Analysis of the digestion of $Man_5GlcNAc_1$ (5a) by the human lysosomal α-mannosidase

(*a*) H.p.l.c. of the digestion mixtures at different time intervals. (*b*) H.p.l.c. of $Man_4GlcNAc_1$ intermediate after acetolysis.

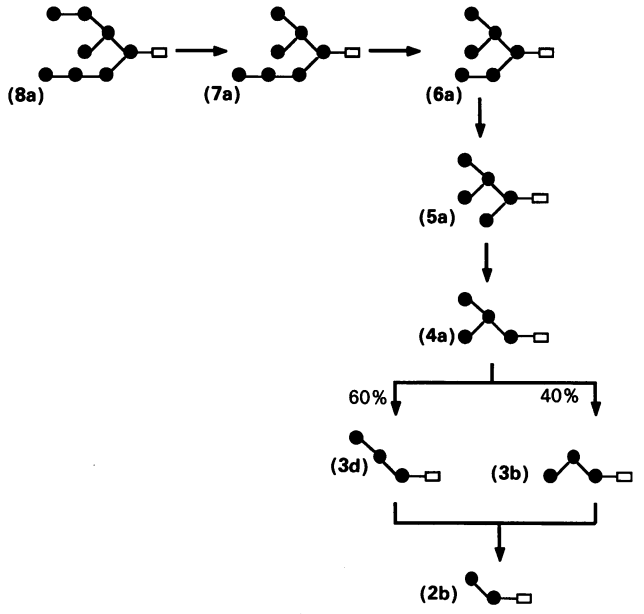
Table 3. Relative rates of hydrolysis of natural substrates by human liver lysosomal α-mannosidase

A 15 nmol portion of each substrate was incubated with 100 munits of lysosomal α-mannosidase for a period of 4 h. The distribution of radioactivity (%) in each intermediate was determined by h.p.l.c., after processing of the samples.

Substrate	Digestion intermediates (% of the starting material)								
	M_9G_1	M_8G_1	M_7G_1	M_6G_1	M_5G_1	M_4G_1	M_3G_1	M_2G_1	M_1G_1
$Man_9GlcNAc_1$ (**9**)	37	42	17	4	0	0	0	0	0
$Man_8GlcNAc_1$ (**8a**)	24	45	30	1	0	0	0	0	0
$Man_5GlcNAc_1$ (**5a**)	–	–	–	–	74	26	0	0	0
$Man_5GlcNAc_1$ (**5b**)	–	–	–	–	35	52	12	0	0
$Man_4GlcNAc_1$ (**4b**)	–	–	–	–	–	69	22	9	0
$Man_4GlcNAc_1$ (**4c**)	–	–	–	–	–	22	61	12	5
$Man_3GlcNAc_1$ (**3a**)	–	–	–	–	–	–	75	22	3
$Man_3GlcNAc_1$ (**3c**)	–	–	–	–	–	–	20	49	31



Scheme 3.



Scheme 4.

5*b*). This provided further strong support for the postulated catabolic route for $Man_8GlcNAc$ (**8a**).

Storage products from mannosidosis. The most abundant storage products in the urine of human patients with mannosidosis consist of a series of straight-chain oligosaccharides (**2a**, **3c** and **4c**) (Scheme 5*a*). Analysis of digestion mixtures by h.p.t.l.c. showed that they are digested very rapidly *in vitro* by lysosomal α-mannosidase (Table 3). None of them is an intermediate in the catabolic pathways for the digestion of high-mannose, complex or hybrid glycans. The addition of an α(1–6)-linked mannose residue to the core β-linked mannose residue (compound **5b**) does not affect the preferential removal of the α(1–2)-linked mannose residues (Scheme 5*b*). The α(1–3)-linked mannose residue is removed preferentially from the core tetrasaccharide, as had been observed previously.

DISCUSSION

The digestion of all the oligosaccharide substrates by lysosomal α-mannosidase is by discrete non-random pathways. It is interesting to compare the structures of the digestion intermediates with the structures of oligomannosides on intact glycoproteins and with those of the storage products in mannosidosis. The unique $Man_9GlcNAc$ oligosaccharide substrate is only found in trace amounts in the urine of patients with mannosidosis. If it is assumed that this is a reflection of its true abundance in intact glycans on glycoproteins, it can be concluded that the vast majority of human asparagine-linked glycans undergo processing.

The $Man_8GlcNAc$ isomer (**8b**) produced by the digestion of $Man_9GlcNAc$ by lysosomal α-mannosidase (Scheme 3) is different from both the major (**8a**) and the minor $Man_8GlcNAc_2$ asparagine-linked glycans found in human IgM (Chapman & Kornfeld, 1979*a*,*b*), which are presumably formed by the action of a human processing α-mannosidase(s). However, isomer (**8b**) is excreted in the urine of patients with mannosidosis and accounts for 20–50% of the $Man_8GlcNAc$ fraction (Yamashita *et al.*, 1980; Matsuura *et al.*, 1981; Egge *et al.*, 1982). This indicates that this structure (**8b**) does occur on intact glycoproteins. If the human endoplasmic-reticulum α-mannosidase has the same specificity as that of the rat (Bischoff *et al.*, 1986), it is tempting to speculate that $Man_8GlcNAc$ (**8b**) is produced by the action of a Golgi-apparatus α-mannosidase I (Tabas & Kornfeld, 1979). Similarly the unique $Man_7GlcNAc$ (**7c**) and

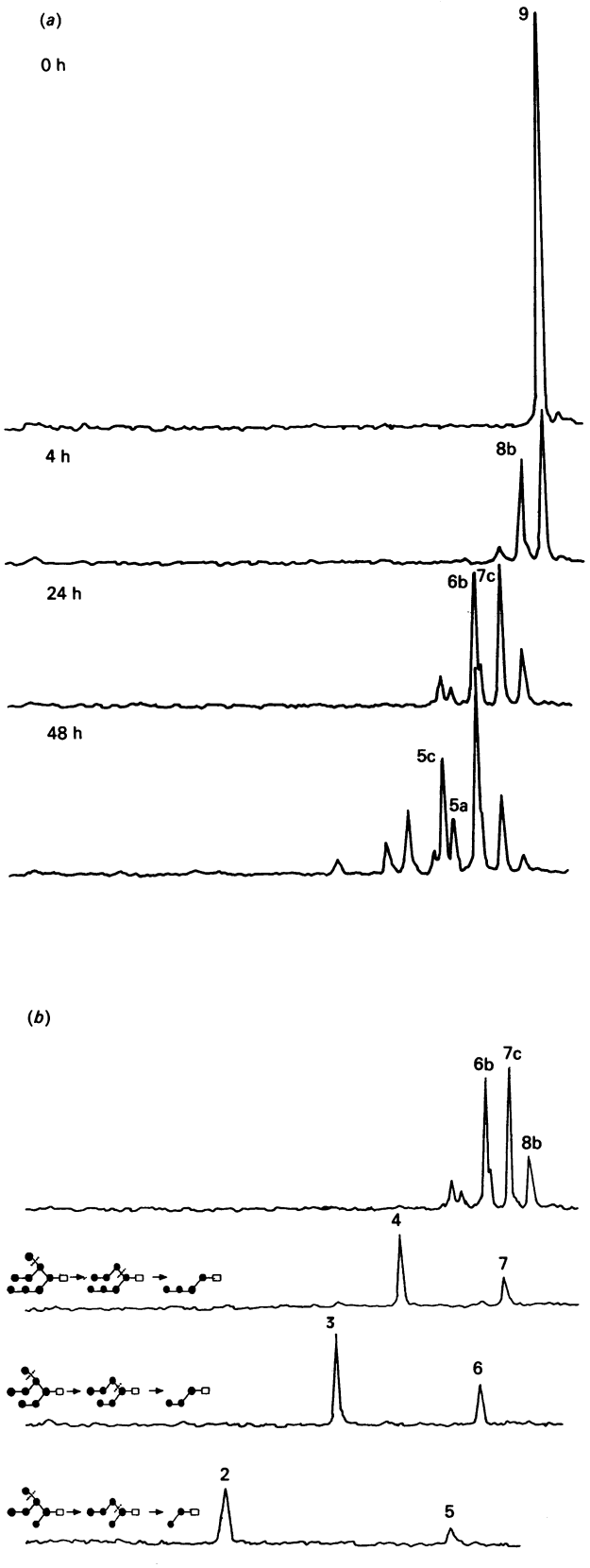


Fig. 4. Analysis of the digestion of $Man_9GlcNAc_1$ (9) by the human lysosomal α-mannosidase

(*a*) H.p.l.c. of the digestion mixtures at different time intervals. (*b*) Acetolysis of the digestion intermediates of composition $Man_8GlcNAc_1$, $Man_7GlcNAc_1$ and $Man_6GlcNAc_1$ isolated by h.p.l.c.

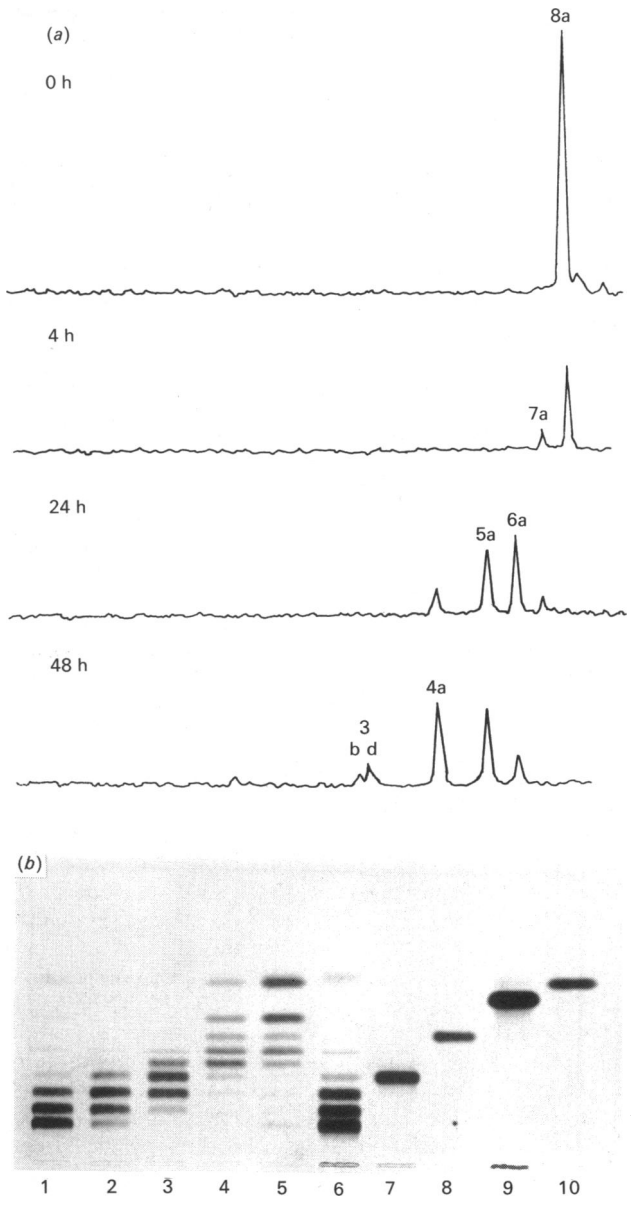
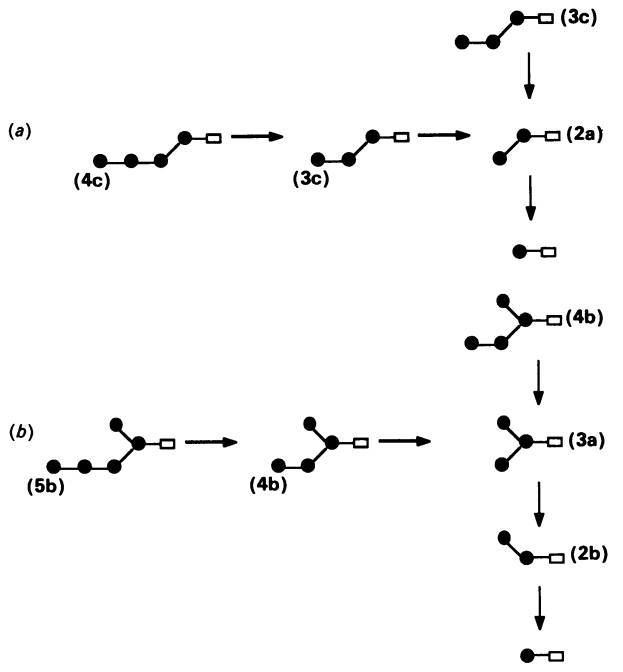


Fig. 5. Analysis of the digestion of $Man_8GlcNAc_1$ (8a) by the human lysosomal α-mannosidase

(*a*) H.p.l.c. of the digestion mixtures at different time intervals. (*b*) H.p.t.l.c. analysis of the digestion of oligosaccharide mixture ($Man_{5-8}GlcNAc$) by the human lysosomal α-mannosidase. Lane 1, 0 h; lane 2, 4 h; lane 3, 6 h; lane 4, 24 h; lane 5, 48 h; lane 6, starting material; lane 7, $Man_5GlcNAc$ (**5a**); lane 8, $Man_3GlcNAc$ (**3a**); lane 9, $Man_2GlcNAc$ (**2a**); lane 10, $Man_1GlcNAc$.

$Man_6GlcNAc$ (**6b**) compounds produced from the digestion of $Man_9GlcNAc$ account for 30–40% and 20–30% of the $Man_7GlcNAc$ and $Man_6GlcNAc$ fractions respectively of the storage products, and are minor and major glycans respectively in human IgM. The predominant $Man_5GlcNAc$ (**5c**), which lacks the core α(1–3)-linked mannose residue, is not present among the storage products or intact glycans. This is not surprising because the core α(1–3)-linked mannose residue is not removed by any known processing mannosidases. The second most abundant $Man_5GlcNAc$ digestion product (**5a**) is the same as the predominant $Man_5GlcNAc$ storage product and the major $Man_5GlcNAc_2$ glycan on intact IgM and complement component C3 (Hase *et al.*, 1985). The final minor $Man_5GlcNAc$ digestion



Scheme 5.

product (**5d**) does not occur in the storage products or intact glycans. Again this is not surprising because all α(1–2)-linked mannose residues are removed by the processing α-mannosidases before any α(1–3)- or (1–6)-linked mannose residues.

The $Man_8GlcNAc$ (**8a**) isomer used as a substrate in these studies is itself a major component of the storage products and intact glycans (Scheme 4). It can be presumed to arise by the action of the putative human endoplasmic-reticulum α-mannosidase. The unique $Man_7GlcNAc$ (**7a**), $Man_6GlcNAc$ (**6a**) and $Man_5GlcNAc$ (**5a**) produced by its digestion with lysosomal α-mannosidase are the major isomers of these compositions in the storage products. The $Man_6GlcNAc$ and $Man_5GlcNAc$ components are the major glycans of this composition on IgM, although different $Man_7GlcNAc$ isomers from the digestion intermediate are on this protein. Thus the lysosomal digestion of this $Man_8GlcNAc$ (**8a**) follows a very similar pathway to the glycoprotein processing route.

The tetrasaccharides (**3b**) and (**3d**) produced from the digestion of $Man_5GlcNAc$ (**5a**) both lack the core α(1–3)-linked mannose residue (Scheme 2*a*). As mammalian processing α-mannosidases do not catalyse the hydrolysis of the core α(1–3)-mannosidic linkage, these structures (**3b** and **3d**) are not found in intact glycoproteins or storage products.

The lysosomal catabolism of the core tetrasaccharide (**3a**) derived from complex glycans is very specific (Scheme 1*a*). A deficiency of lysosomal α-mannosidase would be expected to lead to the accumulation of this tetrasaccharide, but it is present in barely detectable amounts in the urine of patients with mannosidosis. Instead the major storage product, about two-thirds of the total, has the structure Manα(1–3)Manβ(1–4)GlcNAc (**2a**), i.e. it lacks the expected core α(1–6)-linked mannose. It has been concluded that the residual α-mannosidase in mannosidosis can hydrolyse this α(1–6)-linkage. Evidence for this conclusion is provided by experiments in which normal and mannosidosis fibroblasts were grown in the presence of the potent lysosomotropic α-mannosidase inhibitor swainsonine (Cenci di Bello *et al.*, 1983). Under these conditions the accumulation of Manα(1–3)[Manα(1–6)]Manβ(1–4)GlcNAc (**3a**) was induced in both cell types. This confirmed the existence of a residual lysosomal α(1–6)-mannosidase that is inhibited by swainsonine but is unaffected in genetic mannosidosis. It is not known whether this activity is due to a mutant enzyme or a distinct lysosomal enzyme. Therefore the massive accumulation of Manα(1–3)-Manβ(1–4)GlcNAc (**2a**) in mannosidosis is consistent with a total deficiency of the major normal α-mannosidase, which has a preference for hydrolysing the core α(1–3)-mannosidic linkage. An α(1–6)-mannosidase could possibly account for the presence of two other major storage products in mannosidosis, namely the straight-chain $Man_3GlcNAc$ (**3c**) and $Man_4GlcNAc$ (**4c**) (Scheme 5), which together account for about 20% of the total urinary storage products. All of the other known storage products in mannosidosis would give rise to one or other of these three straight-chain compounds if the core α(1–6)-mannosidic linkage could be hydrolysed by the α(1–6)-mannosidase irrespective of whether it is terminal, i.e. if the enzyme is both an exo- and an endo-α-mannosidase. It would have an action analogous to chemical acetolysis. The persistence of some of the larger oligosaccharides in mannosidosis could be due to incomplete digestion by the putative α(1–6)-mannosidase, the tissue distribution of the enzyme or the subcellular segregation of some of the glycans and the enzyme. The presence of a second *N*-acetylglucosamine residue at the reducing terminal appears to block the α(1–6)-mannosidase (Scheme 1*b*). The lack of hydrolysis of Manα(1–6)Manβ(1–4)$GlcNAc_2$ (**2′b**) by the major lysosomal α-mannosidase implies that endohexosaminidase acts before hydrolysis of the core α(1–6)-mannosidic linkage in the normal catabolic pathway. It is speculated that the α(1–6)-mannosidase is not normally needed to break down asparagine-linked glycans but has a role in the catabolism of other glycoconjugates containing α(1–6)-linked mannose such as dolichyl pyrophosphate oligosaccharides or the glycosylphosphatidylinositol membrane anchor.

Thus lysosomal α-mannosidase is able to hydrolyse all the α-mannosidic bonds in asparagine-linked glycans. The catabolic pathway for a substrate appears to be determined by its structure. Interaction between an oligosaccharide substrate and the enzyme to form the enzyme–substrate complex is probably via the peripheral mannose residues at the non-reducing terminal because reduction or addition of another *N*-acetylglucosamine residue to the reducing terminal does not affect the catalysis, except for the unnatural substrate $Man_2GlcNAc_2$ (**2′b**). Therefore it is postulated that each substrate forms a unique enzyme–substrate complex, the conformation of which will determine which α-mannosidic bond is placed adjacent to the catalytic functional groups in the active site and hence hydrolysed. Likewise the conformation of the enzyme–substrate complex formed between the product of the first reaction and the enzyme will determine the next bond to be hydrolysed. The formation of successive enzyme–substrate complexes may take place without the substrate leaving the active site. This mechanism would explain the ordered route of catabolism for each substrate. Where alternative routes occur, noticeably with smaller substrates, flexible conformations of similar energy could exist, permitting juxtaposition of more than one bond with the catalytic machinery.

It is interesting to compare the specificities towards oligosaccharide substrates of lysosomal α-mannosidase in two species expressing endohexosaminidase, the rat and man. In contrast with the human liver enzyme, rat kidney lysosomal α-mannosidase has been reported to act very slowly on high-mannose glycans of composition $Man_{5-8}GlcNAc$ (Tulsiani & Touster, 1987). However, each substrate used in that study consisted of a mixture of isomers, and the products of digestion were not identified. The rat kidney enzyme also hydrolysed the $Man_5GlcNAc$ (**5a**), $Man_3GlcNAc$ (**3a**) and the linear manno-

sidosis storage products (**3c** and **4c**) used in our study. Although the α(1–2)-linkages of the linear oligosaccharides were hydrolysed faster than the α(1–3)- and α(1–6)-linkages, as for the human enzyme, the digestion products were not identified. More recently a detailed investigation of the specificity of rat liver lysosomal α-mannosidase towards $Man_9GlcNAc$ (**9**) and the same $Man_5GlcNAc$ (**5a**), also studied by us, has permitted a direct comparison (Michalski *et al.*, 1990). The digestion of $Man_9GlcNAc$ by the human and rat enzymes follows an identical pathway as far as $Man_7GlcNAc$ (**7c**), showing that the two enzymes have very similar catalytic mechanisms. Whereas the human enzyme converts $Man_7GlcNAc$ (**7c**) predominantly into a single isomer of $Man_6GlcNAc$ (**6b**) (see Scheme 3), the rat enzyme generates a mixture of the heptasaccharides (**6a**) and (**6b**). $Man_6GlcNAc$ (**6a**), of which we had an authentic sample, was not detected in human digestions by h.p.l.c. before or after acetolysis. The rat enzyme then generates an equimolar mixture of the two minor $Man_5GlcNAc$ components (**5a** and **5d**) observed in the human digestion, both of which retain the highly susceptible core α(1–3)-mannosidic linkage. Further digestion of these intermediates by the rat enzyme was not detected. However, when the $Man_5GlcNAc$ (**5a**) was employed as the starting substrate in the presence of Zn^{2+}, it was broken down by the same route as that observed for the human enzyme (Scheme 2*a*), except that the proportions of the two tetrasaccharides (**3d** and **3b**) were different and digestion stopped at the trisaccharide (**2b**). The results obtained with the rat enzyme suggest that more than one lysosomal α-mannosidase or active site is involved in the catabolism of *N*-linked glycans. In contrast the preparation of human liver α-mannosidase was able to hydrolyse all the α-mannosidic linkages in these glycans and did not require Zn^{2+}. A difference between species could explain these results. However, it is also possible that more than one α-mannosidase was present in the crude preparations of rat liver lysosomes used, especially when assayed at pH 5.

Thus human liver lysosomal α-mannosidase can hydrolyse all the α-mannosidic linkages in asparagine-linked glycans on glycoproteins, without the addition of Zn^{2+}. The mannose-rich oligosaccharides that accumulate in the absence of this enzyme in α-mannosidosis are postulated to differ from the expected structures because of the presence of an unaffected lysosomal α(1–6)-mannosidase.

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