Carbohydrate Structures of Recombinant Human α-L-Iduronidase Secreted by Chinese Hamster Ovary Cells

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α-L-Iduronidase is a lysosomal hydrolase that is deficient in Hurler syndrome and clinically milder variants. Recombinant human α-L-iduronidase, isolated from secretions of an overexpressing Chinese hamster ovary cell line, is potentially useful for replacement therapy of these disorders. Because of the importance of carbohydrate residues for endocytosis and lysosomal targeting, we examined the oligosaccharides of recombinant α-L-iduronidase at each of its six N-glycosylation sites. Biosynthetic radiolabeling showed that three or four of the six oligosaccharides of the secreted enzyme were cleaved by endo-β-N-acetylgalactosaminidase H, with phosphate present on the sensitive oligosaccharides and L-fucose on the resistant ones. For structural analysis, tryptic and chymotryptic glycopeptides were isolated by lectin binding and reverse phase high pressure liquid chromatography; their molecular mass was determined by matrix-assisted laser desorption-time of flight mass spectrometry before and after treatment with endo- or exoglycosidases or with alkaline phosphatase. Identification of the peptides was assisted by amino- or carboxy-terminal sequence analysis. The major oligosaccharide structures found at each site were as follows: Asn-110, complex; Asn-190, complex; Asn-336, bisphosphorylated (P2Man7GlcNAc2); Asn-372, high mannose (mainly Man9GlcNAc2, some of which was monoglucosylated); Asn-415, mixed high mannose and complex; Asn-451, bisphosphorylated (P2Man,GlcNAc2). The Asn-451 glycopeptide was unexpectedly resistant to digestion by N-glycanase unless first dephosphorylated, but it was sensitive to endo-β-N-acetylgalactosaminidase H and to glycopeptidase A. The heterogeneity of carbohydrate structures must represent the accessibility of the glycosylation sites as well as the processing capability of the overexpressing Chinese hamster ovary cells.

α-L-Iduronidase (EC 3.2.1.76), a lysosomal enzyme that participates in the degradation of dermatan sulfate and heparan sulfate, is deficient in the Hurler, Hurler/Scheie, and Scheie syndromes, collectively known as mucopolysaccharidosis I (1). In the absence of α-L-iduronidase, lysosomal accumulation of partially degraded glycosaminoglycans causes characteristic clinical manifestations that include corneal clouding, skeletal abnormalities, cardiovascular disease, limited joint mobility, and organomegaly. Mental retardation and death in childhood characterize the Hurler syndrome, while intelligence is normal and life span nearly so in the Scheie syndrome. There exist canine and feline forms of α-L-iduronidase deficiency, and a murine form has recently been generated by homologous recombination (2). The disorders have been extensively reviewed, as have recent studies of their molecular basis (1, 3).

Early work in cell culture had suggested that mucopolysaccharidosis I might be amenable to enzyme replacement therapy, since exogenous enzyme could be taken up by receptor-mediated endocytosis and delivered to lysosomes (1). To provide sufficient enzyme, we isolated a stably transfected Chinese hamster ovary (CHO) cell line that synthesized and secreted large amounts of recombinant human α-L-iduronidase (4). The secreted enzyme had properties desirable for replacement purposes, including efficient endocytosis by cultured fibroblasts through a mannose 6-phosphate-dependent system and a 5-day half-life within the cells. When used in replacement trials for the canine model of α-L-iduronidase deficiency, the recombinant human enzyme was taken up to the largest extent by liver, in lesser amounts by lung, kidney, and spleen, and little if at all by brain, cartilage, myocardium, and cornea (5, 6). Similar results were found in replacement trials for the feline model of the disease (7). It is not known whether this distribution is the result of accessibility of the circulating enzyme to tissues, of uptake of the enzyme by specific receptor systems, or of some combination of these factors.

Overexpressing CHO cell lines have been engineered for production of a number of other soluble lysosomal enzymes (8–13). In contrast to normal cultured cells, which secrete very little lysosomal enzyme except in the presence of NH3 or somatotropin amines, engineered CHO lines secrete a substantial fraction of the newly synthesized recombinant enzymes even in the absence of these weak bases (4, 8, 11, 13). Such secretion occurs although the enzymes appear to have the mannose 6-phosphate signal for targeting to lysosomes, as evidenced by mannose 6-phosphate inhibition of their uptake. There is no detailed information on the structures of the carbohydrate constituents of recombinant lysosomal enzymes. We undertook

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The abbreviations used are: CHO, Chinese hamster ovary; ConA, concanavalin A; dMM, deoxymannojirimycin; endo-H, endo-β-N-acetylgalactosaminidase H; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; PNGase-F, peptide N-glycosidase F (N-glycanase); TPCK, l-1-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, N-tosyl-l-lysine chloromethyl ketone; HPLC, high pressure liquid chromatography; Mes, 4-morpholineethanesulfonic acid.
an analysis of the N-linked carbohydrates of secreted α-L-iduronidase to shed light on processing by overexpressing CHO cells as well as to facilitate interpretation of enzyme uptake in vivo. Since the structure of the carbohydrates at each of the six glycosylation sites (Asn residues 110, 190, 336, 372, 415, and 451 (14)) would be more useful for both purposes than the structure of pooled carbohydrates, we used MALDI-TOF mass spectrometry of isolated glycopeptides before and after treatment with glycosidases and phosphatase (15, 16).

EXPERIMENTAL PROCEDURES

Materials—α-L-Iduronidase was collected from secretions of the overexpressing CHO cell line 2.131 and purified to apparent homogeneity as described previously (4). Antiserum to this enzyme was raised in rabbits. Reagents were purchased from the following vendors: "P" from American RGB; "H" from ICN; Exper
t" H" protein labeling mix from NEN Life Science Products; fetal bovine serum and other tissue culture reagents from Life Technologies, Inc., except for methionine-free Dulbecco’s modified Eagle’s medium, which was from Biofluids; TPCCK-treated trypsin and TLCK-treated chymotrypsin, bra
dykinin, heparin-acrylic beads, and agaroase-linked lectins from Sigma; Pansorbin, Escherichia coli alkaline phosphatase, and Vibrio cholerae neuraminidase from Calbiochem; recombinant endo-N-acetylglu
cosaminidase H (endo-H) from New England Biolabs; deoxyvienm

-Mannose-treated lectin (dMM) from Genzyme; N-glycanase (PNGase-F), V. cholerae sialidase, and α-mannosidase from Oxford Glycosystems; glycopepti
dase A from Seikagaku; α-cyano-4-hydroxycinnamic acid from Aldrich; sequencing grade carbohydrate peroxidase Y and pyrogallamate aminopeptidase from Boehringer Mannheim. α-Glucosidase II was a gift from Drs. Alan Elbein and Y. Zeng of the University of Arkansas for Medical Sciences. Centricron-30 microcentrators were from Amicon, and Sep

-Pak C-18 cartridges were from Waters.

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the presence of dMM, an inhibitor of the Golgi explanation for the incomplete hydrolysis by PNGase-F will be completely, by PNGase-F (Fig. 1 secreted enzyme by endo-H could be cleaved, although not former was completely cleaved by endo-H (Fig. 1 intracellular enzyme differed from secreted enzyme in that the ratio of high mannose to complex oligosaccharides. Newly made intracellular enzyme (Fig. 1 lanes 7 and 8), suggesting that it was present solely on N-linked complex oligosaccharides. Analysis of α-Methylmannoside-eluted Glycopeptides of Secreted α-L-Iduronidase by MALDI-TOF Mass Spectrometry—Fig. 3, A and C, shows the HPLC profile of α-L-Iduronidase glycopeptides produced by trypic and chymotryptic digestion.

**RESULTS**

**Carbohydrate Structures of Metabolically Radiolabeled α-L-Iduronidase—Susceptibility of radiolabeled α-L-Iduronidase to cleavage by endo-H and PNGase-F provided information on the ratio of high mannose to complex oligosaccharides. Newly made intracellular enzyme differed from secreted enzyme in that the former was completely cleaved by endo-H (Fig. 1A, lanes 1 and 3), whereas secreted enzyme was partially resistant (Fig. 1A, lanes 7 and 9). The carbohydrate chains not removed from the secreted enzyme by endo-H could be cleaved, although not completely, by PNGase-F (Fig. 1A, lanes 11 and 12); a possible explanation for the incomplete hydrolysis by PNGase-F will be provided below. Only after the labeling had been performed in the presence of dMM, an inhibitor of the Golgi α-1,2-mannosidase I and hence of the mannose trimming required for complex sugar formation (22), were the migration and endo-H susceptibility of the secreted enzyme equal to those of newly made intracellular enzyme (Fig. 1A, lanes 2, 4, 8, and 10). Thus, the difference is due to complex oligosaccharides on the secreted enzyme.

Intermediate deglycosylation steps were made visible by partial endo-H digestion. Treatment of newly made intracellular α-L-Iduronidase with a reduced amount of endo-H gave a ladder of six distinct bands, not including the starting material (Fig. 1B, left panel), showing that all six sites were of the high mannose type. Similar treatment of the secreted enzyme with endo-H resulted in only 3 or 4 bands (Fig. 1B, right panel), indicating that two or three sites had been processed to the complex form. The bands derived from the secreted enzyme were diffuse and poorly resolved, although the sample had been pretreated with neuraminidase to reduce heterogeneity due to different degrees of sialylation. The diffuse appearance and poor resolution of the bands are attributed to incomplete desialylation and/or glycoform heterogeneity of secreted α-L-Iduronidase.

When the secreted α-L-Iduronidase was biosynthetically labeled with [35S]methionine, all of the phosphate label could be removed by treatment with endo-H (Fig. 2A, lanes 1–4), indicating that it was associated with the carbohydrate and not with the protein. The deglycosylated protein itself could be visualized with [35S]methionine labeling (Fig. 2B, lanes 5–8), showing that it was present with good resolution of the bands are attributed to incomplete deglycosylation and endo-H, and that all six sites were of the high mannose type. Similar treatment of the secreted enzyme with endo-H resulted in only 3 or 4 bands (Fig. 2A, lanes 1–4). The secreted enzyme became strongly labeled after continuous exposure of the cells with [35S]methionine for 6 or 10 h. C, secreted α-L-Iduronidase was immunoprecipitated from the medium after continuous labeling of the cells with [3H]fucose for 12 h; it was treated with endo-H or PNGase-F as indicated.

**Fig. 2. Incorporation of radioactive phosphate and 1-fucose into α-L-Iduronidase.** A, CHO 2.131 cells were exposed to medium containing [33P], (lanes 1–4) or [35S]methionine (lanes 5–8) in the presence or absence of dMM; secreted α-L-Iduronidase was immunoprecipitated and treated with endo-H as indicated. B, α-L-Iduronidase was immunoprecipitated from cells or medium, as indicated, after continuous labeling of the cells with [33P], for 6 or 10 h. C, secreted α-L-Iduronidase was immunoprecipitated from the medium after continuous labeling of the cells with [3H]fucose for 12 h; it was treated with endo-H or PNGase-F as indicated.
Carbohydrate Structures of Recombinant α-L-Iduronidase

Fig. 3. HPLC elution of α-L-iduronidase glycopeptides. Glycopeptides from tryptic (A and B) and chymotryptic (C and D) digests of secreted α-L-iduronidase were eluted from ConA-agarose with α-methylmannoside and separated by HPLC. The top panels show absorbance of the glycopeptides at 215 nm; the bottom panels show 32P radioactivity. The glycopeptides in panel A and the radioactive glycopeptides in panel B are derived from similar but separate incubations with trypsin, whereas the glycopeptides in panel C and the radioactive glycopeptides in panel D come from the same incubation with chymotrypsin.

and isolated by α-methylmannoside elution from ConA-agarose; Fig. 3, C and D, show the corresponding radioactivity profile of 32P-labeled α-L-iduronidase glycopeptides similarly treated with trypsin and chymotrypsin, respectively.

MALDI-TOF analysis of HPLC fractions 69–70 of the tryptic digest (Fig. 3A) gave a prominent signal at m/z 3802.8. This signal disappeared when the fractions were treated with endo-H or PNGase-F and was replaced by signals of m/z 2306.2 or 2103.7, respectively. This decrease in mass resulted from the loss of carbohydrate, the structure of which could be deduced from the magnitude of the decrease (Table I). The carbohydrate itself was not detected, since it does not crystallize with the matrix and/or fails to protonate under the conditions used. Treatment of fractions 69–70 with α-mannosidase produced no change in mass, whereas treatment with alkaline phosphatase resulted in a prominent signal at m/z 3644.7. The changes in mass observed upon treatment with endo-H, PNGase-F, and phosphatase were compatible with the structure P2Man7GlcNAc2 (Table I). The 32P radiolabel in fractions 69–70 (Fig. 3B) confirmed the phosphorylated structure of the oligosaccharide. Some minor signals could also be seen in the intact sample, of m/z 3638.9, 3556.7, and 3390.1, compatible with structures P2Man4GlcNAc2, P2Man5GlcNAc2, and P2Man6GlcNAc2; these accounted for ~6%, 6%, and 2% of the total, respectively. Amino- and carboxyl-terminal sequence analyses as well as the mass of the deglycosylated glycopeptide identified it as Val-325 to Tyr-345 (Table II). The phosphorylated high mannose chain was therefore assigned to Asn-336.

Mass spectrometric analysis of HPLC fraction 66 from the tryptic digest (Fig. 3A) showed a series of signals starting with m/z 3693.4 and decreasing in steps of 162 Da to m/z 2719.5 (Fig. 4). This fraction contained no 32P (Fig. 3B). The major signal of m/z 3530.7 was compatible with a structure of Man3GlcNAc2 (Table I), the signals of lower m/z were compatible with progressively fewer mannose residues down to Man3GlcNAc2, and the signal of m/z 3693.4 was presumed to represent a structure with an additional hexose. Treatment with α-glucosidase II reduced the latter to 3530.5 (Fig. 4), identifying the m/z 3693.4 species as Glc3Man3GlcNAc2 (Table I). Treatment with jack bean α-mannosidase resulted in the expected Man3GlcNAc2 (m/z of 2557.7), with some further removal of one or two mannose residues. However, some of the material was digested only to m/z 3368.5; this signal was presumably derived from the loss of 2 uncovered mannose residues from the glycosylated species. Amino- and carboxyl-terminal sequence analysis as well as the mass of the PNGase-F treated glycopeptide of fraction 66 identified it as Phe-369 to Leu-382 (Table II). The unphosphorylated high mannose chain was therefore assigned to Asn-372. The distribution of the glycoforms was as follows: 13% Glc3Man3GlcNAc2, 62% Man3GlcNAc2, 19% Man4GlcNAc2, and 6% Man5GlcNAc2.

Similar glycoforms on Asn-372 peptides with different COOH termini were also found in fractions 48–50, 58–60, and 61–62 (Fig. 3A, Table II). Further analysis of the tryptic digests failed to yield information about any glycosylation site other than Asn-372 and Asn-336. Since endo-H digestion of biosynthetically labeled α-L-iduronidase had predicted at least three glycosylation sites of high mannose structure (Fig. 1B), we reasoned that some tryptic glycopeptides might not crystallize with the matrix used and therefore would not have been detected in the mass spectrometric analysis.

Similar analyses were therefore performed on chymotryptic digests of secreted α-L-iduronidase. The major peak in HPLC fractions 50–51 (Fig. 3C) was also strongly labeled with 32P (Fig. 3D). Analysis of this fraction by MALDI-TOF mass spectrometry revealed a major signal of m/z 3515.0, with additional strong signals of m/z 3254.6, 3092.3, and 2930.6 as well as a number of minor signals (Fig. 5A). After endo-H cleavage of the intact glycopeptides, two strong signals of m/z 2015.4 and 1590.5 appeared, suggesting the presence of two different peptide backbones. This was confirmed by amino-terminal sequence analysis, which indicated a mixture of two peptides, one containing the Asn-372 and the other the Asn-451 glycosylation site. Since the glycoforms at Asn-372 had already been identified (see above), several signals could be unambiguously assigned to that site: m/z 3254.6, 3092.3, and 2930.6 of the intact sample, corresponding to high mannose forms with 9, 8, and 7 mannose residues, and m/z 1590.5 of the endo-H-treated sample. By the process of elimination, the signals of m/z 3515.0 (intact) and m/z 2015.4 (endo-H-treated) could be assigned to the Asn-451 glycosylation site.

Treatment of the intact sample with α-mannosidase confirmed these assignments and also resolved the glycopeptide mixture. The signal of m/z 3515.0 remained unchanged (Fig. 5B), whereas the signals assigned to Asn-372 were replaced by signals of m/z 2279.2 and 2443.4. Phosphatase digestion of the α-mannosidase-treated glycopeptides (after reisolation on a Sep-Pak cartridge) caused the m/z 3515.0 signal to shift to m/z 3433.6 and 3353.8, corresponding to a loss of one and two phosphates, respectively. Therefore, the m/z 3515.0 oligosaccharide on the Asn-451 site could be assigned the structure of P2Man3GlcNAc2.

In contrast to digestion with endo-H, which gave two distinct products (Fig. 5A), digestion of fraction 50–51 with PNGase-F yielded only one new signal, m/z 1387.8, corresponding to the Asn-372 peptide (Fig. 5B); the signal derived from the phosphorylated oligosaccharide at Asn-451, m/z 3512.8, remained untouched by PNGase-F (Fig. 5B).

The unexpected resistance of the phosphorylated glycopeptide at Asn-451 was investigated further (Fig. 6). Fractions 50–51 were first treated with α-mannosidase to reveal the phosphorylated Asn-451 glycopeptide at m/z 3515 (Fig. 6A),
which was resistant to PNGase-F (Fig. 6, B and C); but if the mannosidase-treated mixture was reisolated and exposed to phosphatase, the resulting monophosphorylated and dephosphorylated glycopeptides (m/z 3433 and 3354, respectively) were rapidly hydrolyzed by PNGase-F (Fig. 6, D–F). Resistance of bisphosphorylated glycopeptides to PNGase-F is not a general phenomenon, since the bisphosphorylated glycopeptide at Asn-336 (tryptic fractions 69–70, Table I) was sensitive to

| TABLE I | Examples of structure assignment based on reduction in mass after enzyme treatment |
|----------|----------------------------------------------------------------------------------|
| Residue mass used for calculations: Glc and Man, 162.1; GlcNAc, 203.2; P, 80.0. |
| Fractions | Treatment | Mass [M + H]^+ | Residues removed | ~Δ mass |
| 69–70 | None | 3804.8 | None | 0.8 | 0 |
| | α-Mannosidase | 3804.0 | None | 0.8 | 0 |
| | Phosphatase | 3644.7 | 2P | 160.1 | 160.0 |
| | Endo-H | 2306.2 | P2Man2GlcNAc | 1498.6 | 1497.9 |
| | PNGase-F | 2103.7 | P2Man2GlcNAc2 | 1701.1 | 1701.1 |
| 66 | None | 3530.7 | None | 0.5 | 0 |
| | Phosphatase | 3530.2 | None | 0.5 | 0 |
| | α-Mannosidase | 2557.7 | 6Man | 160.1 | 160.0 |
| | Endo-H | 1867.3 | Man9GlcNAc | 1664.4 | 1662.1 |
| | PNGase-F | 1664.9 | Man9GlcNAc2 | 1865.8 | 1865.3 |

| TABLE II | Summary of glycopeptides |
| Deglycosylated peptides were prepared by PNGase-F digestion, which converts asparagine residues at the carbohydrate-protein junction to aspartic, with a net gain of 1 Da. The junction Asp residues are numbered. Experimentally determined amino acid sequences are underlined (amino terminus) or italicized (carboxyl terminus). Adjacent amino acids in the intact α-L-iduronidase are shown in parentheses. pE, pyroglutamic; M*, oxidized methionine. ND, not determined. |
| Peptides | Mass | Protease | HPLC fraction | ConA elution | Endo-H cleavage | Comments |
| (R)GLSYDFTHLDGYLDLL(R) | 1842.7 | Try | 84 | Me-Glc | − | α |
| (L)SYDFTHLD(L) | 882.6 | Chy | 60–61 | Me-Glc | − | |
| (L)SYDFTHLL(L) | 884.8 | Chy | 57–58 | Me-Man | ND |
| (W)NEPDHHDFDVSMQ(T) | 1791.6 | Chy | 58 | Me-Glc | − | b |
| (W)NEPDHHDFDVSMQQG(F) | 2132.9 | Chy | 54 | Me-Glc | − | |
| (W)NEPDHHDFDVSM*Q(T) | 1806.4 | Chy | 54 | Me-Glc | − | |
| (W)NEPDHHDFDVSM*Q(G) | 1822.7 | Chy | 54 | Me-Glc | − | |
| (K)VIAQHQLLADITSAF(A) | 2103.7 | Try | 69–70 | Me-Glc | − | |
| (K)VIAQHQLLADITSAF(Y) | 2102.9 | Try | 69–70 | Me-Man | + | c |
| (K)VIAQHQLLADITSAF(P) | 1200.0 | Chy | 61–62 | Me-Man | + | |
| (R)FQVNDTRPHQVQ(L) | 1664.8 | Try | 66 | Me-Man | + | d |
| (R)FQVNDTRPHQVL(R) | 1819.7 | Try | 61–62 | Me-Man | + | |
| (F)PDVNTTRPHQV(L) | 1275.0 | Chy | 37–41 | Me-Man | + | |
| (F)PDVNTTRPHQVL(L) | 1404.7 | Chy | 50–51 | Me-Man | + | e |
| (F)PDVNTTRPHQVQ(L) | 1517.4 | Chy | 59–62 | Me-Man | + | |
| (F)PDVNTTRPHQVLQ(L) | 1500.6 | Chy | 59–62 | Me-Man | + | |
| (W)AEVSQAGTVLSDUHGTGVL(A) | 1897.9 | Chy | 61–62 | Me-Man | + | |
| (T)VLSD | 1645.9 | Chy | 58 | Me-Glc | − | f |
| (Y)ASDDTRAPDRSVAT(T) | 1597.0 | Chy | 39–41 | Me-Man | + | |
| (Y)ASDDTRAPDRSVAT(L) | 1699.3 | Chy | 37–38 | Me-Man | + | |
| (Y)ASDDTRAPDRSVATV(R) | 1810.8 | Chy | 50–51 | Me-Man | + | g |
| (Y)ASDDTRAPDRSVATVRL | 1811.9 | Chy | 51–52 | Me-Glc | −/+ | h |

α Glycopeptide data in Fig. 7.  
b Found in mixture with Asn-415 peptide.  
c Glycopeptide data in Table I.  
d Glycopeptide data in Fig. 4 and Table I.  
e Glycopeptide data in Fig. 5; mixture with Asn-451 glycopeptide.  
f Peptide seen in mixture with Asn-190 peptide by amino-terminal sequence analysis; not seen in mass spectrometry.  
g Glycopeptide data in Fig. 5; mixture with Asn-372 glycopeptide.  
h Endo-H-cleavable oligosaccharide same as that of methylmannoside-eluted glycopeptide.
PNGase-F action without prior dephosphorylation (Fig. 6, G–I). The PNGase-F-resistant glycopeptide was readily cleaved by glycopeptidase A (not shown).

Some Asn-372 glycopeptides in chymotryptic fractions 50–51 showed doublet signals differing in mass by 17 Da; difficulty in obtaining amino-terminal sequence occurred when the smaller member of the doublet predominated. The problem was traced to cyclization of the amino-terminal glutamine to pyroglutamate with loss of NH₃ (23). The presence of pyroglutamate was demonstrated by the reduction in mass by 111 Da when the PNGase-F-deglycosylated peptide was treated with pyroglutamate aminopeptidase (data not shown). Additional cyclized peptides were also found (Table II).

Analysis of Tryptic and Chymotryptic Complex Glycopeptides—Tryptic and chymotryptic glycopeptides eluted from ConA-agarose with α-methyl glucoside were subjected to similar separation on HPLC and analysis by MALDI-TOF mass spectrometry before and after treatment with sialidase or PNGase-F. These fractions would be expected to contain only biantennary complex structures. Assignment of one or more N-acetyl neuraminic acid residues was made on the basis of mass before and after treatment with sialidase. Amino- and carboxyl-terminal amino acid sequence analysis confirmed the identity of the peptides. Fig. 7 shows a glycopeptide from tryptic fraction 84, of mass assignable to Gly-106 to Leu-121 and therefore containing glycosylation site Asn-110. The carbohydrate structure is compatible with NeuAc0–2Gal2GlcNAc4-Man3Fuc1. Similar structures were also observed in methylglucoside-eluted chymotryptic fractions 54 (Asn-190), 58 (Asn-190 mixed with Asn-415), 51–52 (Asn-451), and 60–61 (Asn-110) (not shown). Analysis of the structure of the Asn-415 oligosaccharide is incomplete, since we were not able to resolve it from that of the admixed Asn-190 glycopeptide (Table II).

Attempted analysis of HPLC fractions eluted from wheat germ agglutinin-agarose or castor bean agglutinin-agarose did not provide any identifiable signals on mass spectrometry. Glycopeptides present in these fractions would have contained more branched complex structures, with additional neuraminic acid residues, and may have been undetectable under the conditions used for mass spectrometry.

Summary of Glycosylation Site Assignments—Table II summarizes the data identifying the major glycopeptides found in HPLC fractions of tryptic and chymotryptic digests. The peptide mass after PNGase-F deglycosylation and its amino- and/or carboxyl-terminal amino acid sequence formed the basis of glycosylation site assignment. Analysis of the glycoforms has been described above for the major fractions.

DISCUSSION

Glycosylation of the secreted recombinant α-L-iduronidase is shown schematically in Fig. 8. The structural analyses showed that all of the glycoforms at Asn-372, most glycoforms at Asn-336 and Asn-451, and some unknown fraction at Asn-415 are of the high mannose or phosphorylated high mannose type and therefore endo-H-sensitive. This is in good agreement with the results of biosynthetic experiments, which showed that three or four of the six glycosylation sites were occupied by endo-H-sensitive oligosaccharides. The biosynthesis experiments were carried out under conditions (medium composition and 12-h collection time) that simulated the preparative protocol.

The presence of phosphorylated and unphosphorylated high phosphorylated high mannose oligosaccharides in chymotryptic fractions 50–51 (Asn-451 and Asn-372). HPLC fractions 50–51 from the chymotryptic digest shown in Fig. 3C were subjected to MALDI-TOF mass spectrometry either directly ("intact") or after treatment with α-glucosidase II, α-mannosidase, endo-H, or PNGase-F as indicated. The peptide backbone was identified by the mass of the PNGase-F-treated product as well as by amino acid sequence analysis.

Summary of Glycosylation Site Assignments—Table II summarizes the data identifying the major glycopeptides found in HPLC fractions of tryptic and chymotryptic digests. The peptide mass after PNGase-F deglycosylation and its amino- and/or carboxyl-terminal amino acid sequence formed the basis of glycosylation site assignment. Analysis of the glycoforms has been described above for the major fractions.
mammalian oligosaccharides, as well as of partially desialylated complex oligosaccharides, suggests that α-L-iduronidase could be endocytosed by the widely distributed mannose 6-phosphate receptor, by the mannose receptor of macrophages, or by the asialoglycoprotein receptor of hepatocytes. In addition, antibodies are produced against the administered enzyme, immune complexes could be taken up by the Fc receptor of macrophages. On the other hand, the recombinant α-L-iduronidase did not appear to have the α-L-fucosyl-threonine that has been proposed as a recognition signal (24) and that is known to be a component of complex chains at these positions suggests that in the race between phosphorylation and mannose trimming, the phosphorylation reaction predominated most of the time. Therefore, the absence of any phosphorylated oligosaccharide at Asn-372 indicates that this site was not accessible to the GlcNAc1-phosphotransferase that catalyzes the first step in synthesis of that signal.

It was surprising to find Glc(3)Man(9)GlcNAc(2), accounting for about 13% of the oligosaccharides on Asn-372. We are not aware of other instances of such glucosylated structures in secreted proteins. The single glucose residue is thought to be part of a quality control system that keeps deglucosylating and reglucosylating newly synthesized glycoproteins to ensure their retention in the endoplasmic reticulum until folding is completed (26). The cause of incomplete deglucosylation of the secreted recombinant α-L-iduronidase is not known, but it could be a result of its overexpression.

The CHO cell line used here had been shown to secrete half of the recombinant α-L-iduronidase that it synthesized (4). This propensity for secreting a large fraction of overexpressed lysosomal enzymes makes engineered CHO cells particularly useful, since secreted enzymes are relatively easy to purify. We unexpectedly found another advantage of using secreted enzyme for preparative purposes; only the secreted α-L-iduronidase contained phosphorylated oligosaccharides. Presumably, the intracellular enzyme of CHO cells was dephosphorylated soon after reaching lysosomes, whereas the secreted form was not exposed to phosphatases or protected by Pi present in the medium.

The natural history of recombinant α-L-iduronidase in CHO cells is different from that of the enzyme in diploid human fibroblasts. Fibroblasts secrete very little α-L-iduronidase except in the presence of NH₃ (27, 28); furthermore, the intracellular enzyme retains phosphate groups (27). Because of the very low abundance of α-L-iduronidase in fibroblasts, no detailed structural analyses are available. Our concepts of the biosynthesis and targeting of soluble lysosomal enzymes (e.g. Ref. 29) are based on naturally occurring enzymes, including α-L-iduronidase; they do not explain why or how these processes differ for recombinant enzymes made by transfected cells.

A high degree of intrasite heterogeneity was found in the glycopeptides isolated from proteolytic digests of the secreted human α-L-iduronidase. Intrasite heterogeneity of the oligosaccharides (Figs. 4, 5, and 8) can be ascribed to the carbohydrate processing capabilities of the CHO cells. On the other hand, the intrasite heterogeneity of the peptides (Table II) must be ascribed to sample preparation. Most of the tryptic peptides had carboxyl termini at other than trypsin cleavage

| C     | m     | C     | m     |
|-------|-------|-------|-------|
| P     | m     | P     | c     |
sites, indicating the action of peptidase or protease contaminants in trypsin; the chymotryptic peptides showed alternate cleavage sites due to the broad specificity of chymotrypsin. Cleavage of amino-terminal glutamine residues and oxidation of methionine residues added to the complexity of the mixtures. Since this problem of peptide heterogeneity may be intrinsic to the procedures used, assignment of glycosylation sites based solely on peptide mass and presumptive protease cleavage sites is likely to lead to errors. Sequence of one or both termini of the peptide is needed for correct assignment.

The protocol used here was least successful in the detection and analysis of complex carbohydrates; no tri- or tetra-antennary structures were found in mass spectrometry, and even the biantennary structures gave broad peaks before desialylation. A more complete study of the complex glycopeptides of the secreted enzyme was not attempted; the known processing pathways make it likely that the more highly branched structures would exist only at the sites at which we had identified the biantennary forms. Analysis of purified α-L-iduronidase secreted by the same CHO cells (under slightly different conditions), using fluorophore-assisted carbohydrate electrophoresis (30), had shown a preponderance of triantennary and tetra-antennary complex oligosaccharides.2

In the course of this study, we found that PNGase-F was unable to remove the bisphosphorylated high mannose oligosaccharide from Asn-451, although it readily removed it after partial or complete dephosphorylation. This does not represent a general inability to hydrolyze bisphosphorylated high mannose structures, since the same structure on Asn-336 was sensitive to PNGase-F. Perhaps some interaction between the two phosphate groups and the basic residues surrounding Asn-451 hindered the action of PNGase-F, although the glycopeptide could be cleaved by endo-H and by glycopeptidase A. Whatever the mechanism of PNGase-F resistance, this finding should prompt some caution in interpreting the results of PNGase-F action on glycoproteins bearing phosphorylated carbohydrate chains.

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REFERENCES
1. Neufeld, E. F., and Muenzer, J. (1995) in The Metabolic and Molecular Bases of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 2465–2494, McGraw-Hill Inc., New York
2. Clarke, L. A., Russell, C., Warrington, C., Pownall, S., Borowski, A., Jirik, E. R., Toone, J., and Dimmick, J. (1996) Am. J. Hum. Genet. 59, A196
3. Scott, H. S., Bunge, S., Gal, A., Clarke, L. A., Morris, C. P., and Hopwood, J. (1995) Hum. Mutat. 6, 288–302
4. Kakkin, E. D., Matynia, A., Jonas, A. J., and Neufeld, E. F. (1994) Protein Exp. Purif. 5, 225–232
5. Shull, R. M., Kakkin, E. D., McEntee, M. F., Kania, S. A., Jonas, A. J., and Neufeld, E. F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12937–12941
6. Kakkin, E. D., McEntee, M. F., Schmidtschen, A., Neufeld, E. F., Ward, D. A., Gompf, R., Kania, S., Bedolla, C., Chien, S. L., and Shull, R. M. (1996) Biochem. Mol. Med. 58, 156–167
7. Haskins, M. E., Kakkin, E. D., Wan, O., Weil, M. A., Aguirre, G. D., and Schuchman, E. H. (1995) Am. J. Hum. Genet. 57, A30
8. Ioannou, Y. A., Bishop, D. F., and Desnick, R. J. (1992) J. Cell Biol. 119, 1137–1150
9. Anson, D. S., Taylor, J. A., Bielicki, J., Harper, G. S., Peters, C., Gibson, G. J., and Hopwood, J. J. (1992) Biochem. J. 284, 789–794
10. Bielicki, J., Hopwood, J. J., Wilson, P. J., and Anson, D. S. (1993) Biochem. J. 289, 241–246
11. Ling, P., and Roberts, M. (1993) Biol. Reprod. 49, 1317–1327
12. Unger, E. G., Durrant, J., Anson, D. S., and Hopwood, J. J. (1994) Biochem. J. 304, 43–49
13. Van Hove, J. L. K., Yang, H. W., Wu, J. Y., Brady, R. O., and Chen, Y. T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 65–70
14. Scott, H. S., Anson, D. S., Orsborn, A. M., Nelson, P. V., Clements, P. R., Morris, C. P., and Hopwood, J. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9695–9699
15. Huberty, M. C., Vath, J. E., Yu, W., and Martin, S. A. (1993) Anal. Chem. 65, 2793–2800
16. Sutton, C. W., O’Neill, J. A., and Cottrell, J. S. (1994) Anal. Biochem. 218, 34–46
17. Zhao, K. W., Stevens, B. L., Faull, K. F., Kakkin, E. D., and Neufeld, E. F. (1996) FASEB J. 10, A1108
18. Proia, R. L., D’Azzo, A., and Neufeld, E. F. (1984) J. Biol. Chem. 259, 3359–3354
19. Kaemml, U. K. (1970) Nature 221, 680–685
20. Kaushal, G. P., Pastuszak, I., Hatanaka, K., and Elbein, A. D. (1990) J. Biol. Chem. 265, 16271–16279
21. Patterson, D. H., Tarr, G. E., Regnier, F. E., and Martin, S. A. Anal. Chem. 67, 369–384
22. Elbein, A. D. (1991) FASEB J. 5, 3055–3063
23. Khankle, K. M., Fairwell, T., Chait, B. T., and Manjula, B. N. (1995) Int. J. Peptide Protein Res. 44, 118–123
24. Hajjar, K. A., and Reynolds, C. M. (1993) J. Clin. Invest. 93, 703–710
25. Stults, N. L., and Cummings, R. D. (1993) Glycobiology 3, 589–596
26. Peterson, J. R., Ora, A., Van, P. N., and Helenius, A. (1995) Mol. Biol. Cell 9, 1173–1184
27. Myerowitz, R., and Neufeld, E. F. (1981) J. Biol. Chem. 256, 3044–3048
28. Taylor, J. A., Gibson, G. J., Brooks, D. A., and Hopwood, J. J. (1991) Biochem. J. 274, 265–268
29. Korndfeld, S. (1987) FASEB J. 6, 462–468
30. Starr, C. M., Massada, R. L., Hague, C., Skop, E., and Klock, J. C. (1996) J. Chromatogr. 720, 295–321

2 C. Starr and C. Hague, personal communication.