Limited Proteolysis of the $\beta$-Hexosaminidase Precursor in a Cell-free System*

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Pulse-chase experiments had shown that $\beta$-hexosaminidase was synthesized in cultured human fibroblasts in precursor form and that during maturation of the enzyme the $\alpha$-chains were converted from $M_\text{r}=97,000$ to 54,000 and the $\beta$-chains from $M_\text{r}=63,000$ to 29,000 plus smaller fragments, probably through an intermediate form of $M_\text{r}=52,000$ (Hasilik, A., and Neufeld, E. F. (1980) J. Biol. Chem. 255, 4937-4945). The $\beta$-hexosaminidase precursor which is present in NH₄⁺-induced secretions has now been used as substrate to study these conversions in a cell-free system. A concentrate of such secretions, labeled biosynthetically, was incubated with cell fractions or purified proteinases; after the reaction, the $\beta$-hexosaminidase was immunoprecipitated and its constituent polypeptides were denatured, reduced, and analyzed by polyacrylamide gel electrophoresis and fluorography.

A $3\times 10^{12}$ $\times$ fraction of fibroblasts catalyzed the conversion of the $\alpha$-chain of the precursor $\beta$-hexosaminidase from $M_\text{r}=67,000$ to 56,000 and of the $\beta$-chain from $M_\text{r}=63,000$ to 63,000. These products were similar to the mature $\alpha$-chain and the intermediate form of the $\beta$-chain. The reaction occurred at acid pH, was stimulated by dithiothreitol, and was inhibited by iodoacetamide, N-ethylmaleimide, leupeptin, chymostatic, and antipain. Phenylmethylsulfonyl fluoride gave partial inhibition whereas EDTA, pepstatin A, and aprotinin had no effect. These properties indicate that the reaction was catalyzed by a lysosomal thiol proteinase; however, the enzyme was probably distinct from cathepsin B, as purified cathepsin B itself had no effect on the $\beta$-hexosaminidase precursor.

A similar reaction was also catalyzed by trypsin, chymotrypsin, and some related proteinases. Very high levels of trypsin released in addition polypeptides of $M_\text{r}=50,000$ and lower molecular weight, which resembled the products of intracellular $\beta$-chain maturation.

Formation of the 56,000 and 53,000 products by the lysosomal fraction, trypsin, and chymotrypsin proceeded equally well if the radioactive $\beta$-hexosaminidase precursor was used as substrate in the form of an immunoprecipitate; the remainder of the precursor molecule appeared to be degraded. The limited proteolysis of the $\beta$-hexosaminidase precursor did not affect its catalytic activity toward a synthetic substrate or its potential for uptake mediated by the mannose 6-phosphate recognition system.

Lysozomal enzymes are generally synthesized as precursors with apparent molecular weights higher than those of the mature forms. This has been found true of $\beta$-galactosidase of mouse peritoneal macrophages (1), of $\beta$-hexosaminidase, $\alpha$-glucosidase, cathepsin D (2), and $\alpha$-I-iduronidase (3) of cultured human fibroblasts, of cathepsin D of cultured porcine kidney (4), of $\beta$-hexosaminidase and $\alpha$-I-iduronidase of Chinese hamster ovary cells (5) and of $\beta$-hexosaminidase of cultured mouse macrophages and rat basophilic leukemia cells (5). In many cases, maturation of the precursor form proceeds through several intermediate steps. Maturation may be relatively fast (about 3 h for cathepsin D in human fibroblasts) or extremely slow (several days for $\alpha$-I-iduronidase in the same cells). The precursors in question should not be confused with the very short-lived $\alpha$-pre forms carrying the signal or leader sequences for insertion of the nascent enzymes into the cisternae of the endoplasmic reticulum (6, 7). Although evidence for such a $\alpha$-pre form currently exists only for cathepsin D (4), the synthesis of all lysosomal enzymes is believed to begin in this way.

The maturation of $\beta$-hexosaminidase within human fibroblasts had been previously reported to involve a change in the apparent molecular weight of the $\alpha$-chain from 67,000 to 54,000, and the $\beta$-chain from 63,000 to 29,000 and smaller fragments, probably through a transient intermediate form of $M_\text{r}=52,000$. These changes were presumed to be due, at least in part, to limited proteolysis. In the present work, we have studied the limited proteolysis of the $\beta$-hexosaminidase precursor in a cell-free system, using a fraction derived from fibroblasts as well as defined enzymes.

MATERIALS AND METHODS

Reagents—$-[$4,5-H]Leucine was purchased from Amersham Radiochemical Centre (40-76 Ci/mmoll or from New England Nuclear (46-50 Ci/mmoll); $-[$35S]Sphinganine (800-910 Ci/mmoll) was purchased from Amersham. C-labeled molecular weight standards were from New England Nuclear. 4-Methylumbelliferyl-2-acetamido-2-deoxy-D-glucoside was purchased from Research Products International. Bovine serum albumin with very low $\beta$-hexosaminidase activity was obtained from Calbiochem. Leupeptin, antipain, and chymostatin were from Peninsula Laboratories, N-ethylmaleimide was from K and K Laboratories; other proteinase inhibitors were from Sigma.

$\beta$-Hexosaminidase and cathepsin D from human placenta had been purified by Dr. A. Hasilik (2). Cathepsin B from porcine liver and parathyroid was a generous gift from Dr. R. MacGregor, University of Kansas School of Medicine, Kansas City, MO. Trypsin and chymotrypsin were purchased from Miles and Schwarz BioResearch, respectively, whereas trypsin treated with diphenylcarbamyl chloride and chymotrypsin treated with tosyl lysine chloromethyl ketone were from Sigma. A proteinase from Myxobacter sp., specific for the carboxyl group of lysine (endoproteinase lys-C) was from Boehringer Mannheim. All other proteinases were from Sigma.

Antiserum had been raised in goats against human placental $\beta$-hexosaminidase A (2).


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Cell Culture—Human diploid fibroblasts were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ, and from samples submitted to this laboratory for diagnosis. They were maintained as previously described (8).

Preparation of NH₄⁺-induced Secretions—Fibroblasts were labeled in 100-mm Petri dishes with 0.4 mCi of [3H]leucine or [35S]-methionine in 150 cm² flasks with 1 ml of labeled amino acid. The labeling was essentially as described (2) but serum was omitted from the medium because its presence in medium concentrates interfering substances. The labeling was essentially as described (2) but serum was omitted from the medium because its presence in medium concentrates interfering substances. For labeling with [35S]-methionine, the unlabeled methionine concentration was adjusted to 0.5 μg/ml and two washes replaced the hour starvation that was used for labeling with [3H]leucine. Ammonium chloride, 10 mM, was present in the medium to enhance the secretion of precursor enzymes (2, 9).

After a labeling period of 8–24 h, 1 mg of bovine serum albumin was added as carrier to each milliliter of medium, and protein was precipitated by addition of (NH₄)₂SO₄ to 85% saturation. The precipitate was dissolved in H₂O (0.1 ml/ml of medium), dialyzed against two baths, 2 liters each, of 0.15 M NaCl, 0.01 M sodium phosphate, pH 6.8, and stored at −20 °C.

Cell Fractionation—Cells were harvested with buffered trypsin, washed with fresh medium, and disrupted by N₂ cavitation, as described (10) except for omission of a post-trypsinization recovery period. Nuclei and cell debris were removed by centrifugation at 3,000 × g for 5 min. A fraction enriched in lysosomes was collected by centrifugation of the postnuclear supernatant at 12,000 × g for 20 min; the pellet was suspended in 0.25 M sucrose containing 0.01 M sodium phosphate, pH 6.8 (0.2 ml for the harvest of 1 flask, 150 cm²). The clarified supernatant was stored at −20 °C. Microsomes were prepared by further centrifugation at 100,000 × g for 1 h and suspended in the same manner.

Limited Proteolysis in Cell-free Systems—A typical reaction mixture contained 25–50 μl of labeled concentrate of NH₄⁺-induced secretions (about 2 × 10⁵ cpm) as substrate; cell fraction, up to 100 μl, or defined enzyme in 50 μl; 0.1 M buffer (citrate-phosphate at pH 4.4, 6.0, or 7.6; sodium phosphate at pH 6.8), 3 mM NaN₃, and other reagents as specified in a total volume of 0.3 ml. At the start, reagents were mixed by brief sonication (10 s in a bath sonicator). After incubation at 37 °C for the indicated time, reaction mixtures at pH 4.4 were neutralized with 187 μl of cold 0.15 M NaOH, 0.15 M NaCl, whereas the neutral mixtures were diluted with the same amount of cold 0.01 M NaOH, pH 6.8, 0.15 M NaCl.

Immunoprecipitation and Electrophoretic Analysis of β-Hexosaminidase—The diluted and neutralized reaction mixtures were treated by addition of 2 μl of 20% Triton X-100, sonication for 5 s, and further addition of 5 μl of 3% pronase, followed by centrifugation at 40,000 × g for 1 h. The clarified supernatant solutions were used for immunoprecipitation with antisera to β-hexosaminidase A (2). Immune precipitates were dissolved in sodium dodecyl sulfate-dithiothreitol and subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate by the method of Laemmli (11) with minor modifications (2); the radioactive bands were visualized by fluorography (12).

14C-methylated molecular weight standards included phosphorylase B, 92,300; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; lysozyme, 14,000; or cytochrome c, 12,500.

**RESULTS**

Limited Proteolysis of the β-Hexosaminidase Precursor by a Cell-free System from Human Fibroblasts—Concentrated radioactive secretions, collected from normal human fibroblasts in the presence of NH₄Cl, were incubated with cell fractions as indicated in Fig. 1; after incubation, the β-hexosaminidase was isolated by immunoprecipitation, and its constituent polypeptides were determined by gel electrophoresis under reducing and denaturing conditions.

The secretions contained two radioactive immunoprecipitable polypeptides, apparent Mᵑ = 67,000 and 63,000 (67K and 63K polypeptides), previously shown to correspond to the α- and β-chains, respectively, of β-hexosaminidase (2). These polypeptides remained intact if the concentrate of secretions, denoted substrate on Fig. 1, was incubated alone for 5 h at pH 4.4 or 7.6. However, if the substrate was mixed with the 3,000–12,000 × g "lysosomal" fraction, two new polypeptide chains of Mᵑ = 56,000 and 53,000 appeared after incubation at pH 4.4 but not after incubation at pH 6.0 or 7.6. No additional bands appeared upon longer incubation (24 h), and no bands of intermediate size were detected in shorter incubations. The alpha-chain (67K) disappeared somewhat faster than the β-chain (63K).

The 12,000–100,000 X g microsomal fraction was inactive at both acid and neutral pH. Addition of ATP and an ATP-regenerating system (carnitine phosphate and creatine phosphokinase) did not affect the results with either fraction.

The 56K2 reaction product was identified as an α-chain and the 53K was identified as a β-chain by using as substrate the labeled secretions of mutant fibroblasts from patients with Sandhoff or Tay-Sachs disease, which had been shown (2) to contain only the α- or β-precursor chains, respectively (Fig. 1).

The precursor derived from NH₄⁺-induced secretions of normal cells could be replaced as substrate by intracellular precursor present in an extract of cells pulse-labeled with [3H]leucine (not shown).

**Inhibitors of the Limited Proteolysis of the β-Hexosaminidase Precursor—Class-specific proteinase inhibitors (13) were used to characterize the activity of the lysosomal fraction which catalyzed the cleavage of β-hexosaminidase precursor. As shown in Fig. 2, iodoacetamide and N-ethylmaleimide were inhibitory, whereas diithiothreitol stimulated the reaction. The microbial peptides, leupeptin, antipain, and chymostatin, 1 The abbreviation used is: 56K, 52K, etc., polypeptides of Mᵑ = 56,000, 52,000, etc.
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which inhibit both thiol and serine proteinases, were effective inhibitors; leupeptin completely abolished the reaction at a concentration as low as 0.3 μg/ml (0.6 × 10⁻⁶ M; not shown). On the other hand, pepstatin A, an inhibitor of carboxyl proteinases such as cathepsin D, did not affect the reaction; neither did EDTA, an inhibitor of metalloproteinases, nor aprotinin, an inhibitor of trypsin-like proteinases (not shown). Phenylmethylsulfonyl fluoride, a potent inhibitor of serine proteinases but also somewhat inhibitory of thiol proteinases, gave partial inhibition.

Growth medium containing fetal bovine serum completely inhibited the reaction presumably because of the presence of α₂-macroglobulin, an inhibitor of many endoproteinases (14).

Cleavage of β-Hexosaminidase Precursor Chains by Defined Proteinases—Chymotrypsin, at concentrations ranging from 1-50 μg/reaction, and trypsin, 10 μg/reaction, also catalyzed the conversion of β-hexosaminidase precursor chains to products of Mᵦ = 56,000 and 53,900 (Fig. 3). Pancreatic kallikrein and plasmin, proteinases with trypsin-like specificity, gave similar patterns (not shown). At 50 μg/reaction, trypsin catalyzed the additional formation of a major band at Mᵦ = 30,000 (which appears as a doublet) and of some shorter chains, thus yielding a pattern reminiscent of that of intact cells after a pulse and chase (Fig. 3). The same results were obtained by use of trypsin treated with diphenylcarbamyl chloride and of chymotrypsin treated with tosyl lysine chloromethyl ketone to inactivate contaminating chymotrypsin and trypsin, respectively. Treatment with the lysine-specific endoproteinase of Myxobacter resulted in four distinct bands in the 50K-60K range.

Highly purified cathepsin D failed to degrade the β-hexosaminidase precursor chains (Fig. 3); this had been anticipated from the lack of effect of pepstatin A (a potent inhibitor of cathepsin D) on the lysosomal fraction. On the other hand, the lack of activity of cathepsin B (Fig. 3) was somewhat surprising, because the lysosomal fraction showed properties characteristic of thiol proteinases, and cathepsin B is the major thiol proteinase of lysosomes. The amount of purified cathepsin B selected for testing had the same activity toward benzoylarginine naphthylamide as the lysosomal fraction. Cathepsin B was also inactive toward β-hexosaminidase at pH 5.8 (not shown), under conditions in which it had been shown to catalyze the limited proteolysis of parathormone and parathormone (15).

Other enzymes which appeared to have no effect on the molecular weight of the β-hexosaminidase precursor chains included carboxypeptidases A, B, and Y, as well as cytosolic and microsomal leucine aminopeptidases.

Immunoprecipitated β-Hexosaminidase Precursor as Substrate—The immunoprecipitate of the β-hexosaminidase precursor was a substrate for the lysosomal fraction of fibroblasts, for trypsin, and for chymotrypsin (Fig. 4). In this case, β-hexosaminidase was the only radioactive protein present in the reaction mixture; the results were identical with those seen in the usual protocol of immunoprecipitation after the enzyme reaction, in which case β-hexosaminidase represents less than 1% of the total radioactive material in the substrate preparation.

Immunoprecipitation before treatment allowed a search for the other products of the proteolytic cleavage. For this purpose, the standard procedure was modified to avoid the loss of small polypeptides. [³⁵S]Methionine was substituted for

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**Fig. 2. Effect of proteolysis inhibitors.** The 3,000-12,000 × g lysosomal fraction, 90 μl in 0.25 M sucrose containing 0.01 M NaH₂PO₄, pH 6.8, was preincubated with the inhibitors for 10 min at 37°C in a volume of 100 μl, and then the reaction mixtures were completed for pH 4.4 as indicated under "Materials and Methods." Reactions were incubated further for 5 h at 37°C. The concentrations in the preincubation were as follows, from left to right: iodoacetamide and N-ethylmaleimide (NEM), 2 mM; dithiothreitol (DTT), 3 mM; leupeptin, chymostatin, antipain, and pepstatin A, 100 μg/ml; phenylmethylsulfonyl fluoride (PMSF), 1 mM; EDTA, 3 mM; growth medium containing 10% fetal bovine serum, 70 μl/sample.

**Fig. 3. Limited proteolysis of β-hexosaminidase precursor by defined enzymes.** Incubations were carried out for 5 h as described under "Materials and Methods," with the indicated modifications. From left to right: no addition of protease, pH 6.8; lysosomal fraction, 50 μl, pH 4.4, 3 mM dithiothreitol; trypsin, 10 μg, pH 6.8; diphenylcarbamyl chloride-treated trypsin, 50 μg, pH 6.8; tosyl lysine chloromethyl ketone-treated chymotrypsin, 50 μg, pH 6.8; lysinespecific proteinase of Myxobacter, 20 μg, pH 6.8; cathepsin D, 10 μg, pH 4.4; cathepsin B, 8 μg, pH 4.4, with 3 mM dithiothreitol and 3 mM EDTA. The last lane shows intracellular β-hexosaminidase isolated from cells that had been labeled with [³⁵S]leucine for a 3-h pulse followed by a 19-h chase. The secretions used as substrate were labeled with [³⁵S]methionine in Lanes 4-6 from the left, and with [³⁵S]leucine in all the others.
Fig. 4. Limited proteolysis of immunoprecipitated β-hexosaminidase. Immunoprecipitates of [35S]methionine-labeled β-hexosaminidase precursor were prepared as usual (2) but with the omission of the acetone wash of the precipitates. The immunoprecipitates were incubated for 3 h at 37°C as described under “Materials and Methods” except for a reduction in volume to 40 µl with the following: 1) pH 4.4 with 20 µl of lysosomal fraction, 3 mM dithiothreitol, and 10 µg of leupeptin; 2) pH 4.4 with 20 µl of lysosomal fraction and 3 mM dithiothreitol; 3) pH 6.8 with 10 µg of trypsin; 4) pH 6.8 with 10 µg of chymotrypsin.

TABLE I
Retention of enzyme activity after treatment with proteinases

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<th>Proteinase treatment</th>
<th>β-Hexosaminidase activity</th>
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<tr>
<td>None</td>
<td>4.5</td>
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<tr>
<td>Trypsin, 10 µg</td>
<td>4.6</td>
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<tr>
<td>Trypsin, 50 µg</td>
<td>4.8</td>
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<tr>
<td>Chymotrypsin, 10 µg</td>
<td>4.9</td>
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<td>Chymotrypsin, 50 µg</td>
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[3H]leucine; gels of 15% polyacrylamide were used, dried without prior fixation, and subjected to radioautography rather than fluorography. In spite of these precautions, we failed to detect 10K fragments of the precursor. Radioactive material was found at the dye front after incubation of the immunoprecipitated β-hexosaminidase precursor with the lysosomal fraction and as a heterogeneous mixture of relatively low molecular weight after treatment with trypsin or chymotrypsin.

Enzymatic Activity of β-Hexosaminidase after Limited Proteolysis—The ability of β-hexosaminidase to hydrolyze a fluorogenic substrate, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-glucoside, was not altered upon treatment of the precursor enzyme with trypsin or chymotrypsin (Table I). The change in apparent molecular weight of the β-hexosaminidase chains after treatment with the proteinases has been shown in Fig. 3.

Receptor-mediated Uptake of β-Hexosaminidase after Limited Proteolysis—Fig. 5 compares the endocytosis by cultured fibroblasts of β-hexosaminidase precursor from NH4Cl-induced secretions of the enzyme after partial proteolysis by chymotrypsin and of the enzyme fully processed intracellularly. It can be seen that the enzyme in the three different molecular forms was taken up to the same extent. By summing up the radioactivity recovered from cells and medium (see Fig. 5), the uptake of precursor, chymotrypsin-treated precursor, and intracellularly processed β-hexosaminidase was found to be 20, 24, and 17% of available enzyme, respectively. In each case, uptake of β-hexosaminidase was reduced to 1% or less of available enzyme by the presence of 1 mM mannose 6-phosphate in the medium. The endocytosed precursor and chymotrypsin-treated precursor were correctly converted by the fibroblasts to polypeptides of the size of mature endogenous β-hexosaminidase; no processing occurred extracellularly (Fig. 5).

DISCUSSION
A distinctive fraction of cultured human fibroblasts catalyzed a limited proteolysis of the precursor of β-hexosaminidase to products of Mr ≈ 56,000 and 53,000 for the α- and β-chains, respectively. The active principle functioned at acid...
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pH, was activated by dithiothreitol, and was inhibited by N-ethylmaleimide, iodoacetic acid, leupeptin, chymostatin, and antipain. These properties place the enzyme in the class of lysosomal thiol proteinases which includes cathepsin B, H, L, and N (16). However, cathepsin B, the most abundant of these, did not itself catalyze the conversion of the β-hexosaminidase precursor.

The limited proteolysis of β-hexosaminidase in intact cells probably occurs in the lysosomal apparatus. This has been inferred from the relatively long time (over 5 h) needed to complete maturation in pulse-chase experiments (2) and from the maturation of endocytosed precursor enzyme, which would have been transported to lysosomes (10). Thus, the lysosomal thiol proteinase is a good candidate for the enzyme that would catalyze the initial proteolytic step in intracellular maturation.

The action of the lysosomal enzyme could be duplicated by chymotrypsin and trypsin, and by some related proteinases. At very high concentrations, trypsin catalyzed the further cleavage of the 53K β-chain to 30K and smaller fragments, in a pattern resembling that seen intracellularly.

The lysosomal fraction, trypsin, and chymotrypsin catalyzed the cleavage to the 56K and 53K products even when the β-hexosaminidase precursor was used in the form of a precipitate with its antibody. This procedure purifies the β-hexosaminidase precursor, and the result rules out some indirect effect of the proteinase, such as activation of an unknown enzyme present in the secretions. It also indicates that the antibody (which had been raised against the processed hexosaminidase precursor chains) did not interfere with the limited proteolysis.

The data suggest that the β-hexosaminidase precursor has a segment readily accessible to proteinases, extending about 10,000 daltons from the end of each chain. There is a second area of cleavage roughly in the middle of the β-chain, which appears readily accessible to proteinases in the intact cell but which has proved to be remarkably resistant in cell-free systems. Perhaps proteolysis in that region depends on some prior change, such as removal of carbohydrate groups. It is interesting that, so far, only human β-hexosaminidase has been found to have a β-chain cleavable to 29K. In Chinese hamster ovary cells, cultured mouse macrophages, and rat basophilic leukemia cells, both chains of β-hexosaminidase remain at a molecular weight of over 50,000 (5).

Although we were not able to recover intact fragments of 10K in the cell-free systems, the absence of reaction intermediates between 67K and 56K, or between 53K and 53K, suggests an endoproteolytic attack followed by degradation of the small fragments. Evidence has been presented in other systems for the existence of intact fragments that remain associated with the larger pieces in processed β-hexosaminidase (5). We do not yet know whether the 10K segments are cleaved from the NH₂ or carboxyl termini of the β-hexosaminidase precursor chains. However, they must be removed from the same side by chymotrypsin and by the intracellular system. This is shown by the identical size of the chains that result from the intracellular processing of endogenous enzyme and of enzyme that had been treated with chymotrypsin and subsequently endocytosed.

The synthesis of proteins in precursor form is usually associated with some specific function of the additional sequences (17). We have tested two potential functions of the extra segments of the β-hexosaminidase precursor. The segments appear to play no role in the catalytic activity of the enzyme toward a synthetic substrate (but note that a change in Kₘ might not have been detected under the conditions used and that the natural substrates have not been tested). In addition, the extra segments did not appear necessary for uptake of β-hexosaminidase by the mannose 6-phosphate recognition system (18, 19); β-hexosaminidase that had been partially processed by chymotrypsin or processed fully within the fibroblasts was endocytosed as efficiently as the precursor enzyme. In this respect, β-hexosaminidase differed from α-L-iduronidase, which retained its catalytic activity but lost capacity for uptake after intracellular processing (3). If there exist specific functions for the precursor segments of β-hexosaminidase, they may be fulfilled in the early steps of the formation of the enzyme, e.g. in the alignment of the subunits or in the identification of the protein as a substrate for the synthesis of the mannose 6-phosphate recognition marker.

REFERENCES