Kinetic mechanism and characterization of human β -galactosidase precursor secreted by permanently transfected Chinese hamster ovary cells

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Chinese hamster ovary cell clones permanently transfected with the cDNA for human lysosomal β -galactosidase secrete the enzyme precursor into the cell medium, from which it is purified to apparent homogeneity in a single step by affinity chromatography. The purified precursor is fully active, displays the same pH optimum and K_m values as the mature placental enzyme, and has an intact C-terminus. The intact enzyme when chromatographed on a Sephacryl S-200 molecular-sieve column elutes as a 105 500 Da monomer, whereas on SDS/PAGE gels the polypeptide migrates as an 88 kDa polypeptide. A time course of digestion with glycopeptide-N-glycanase shows the gradual conversion of the precursor from an 88 to a 72 kDa protein, suggesting the presence of five N-linked oligosaccharides in the protein. The precursor is readily taken up in a mannose-6-phosphate-dependent manner into β -galactosidase-deficient,

GM1-gangliosidosis fibroblasts, and the enzyme activity is returned to normal levels. We show that the stereochemical course of enzymic hydrolysis involves the retention of the β -configuration at the anomeric centre, suggesting a double-displacement mechanism. Furthermore, the enzyme is rapidly and irreversibly inactivated in the presence of the mechanism-based inactivator 2,4-dinitrophenyl-2-deoxy-2-fluoro- β -D-galactopyranoside, which implicates a covalent intermediate. The enzyme is also inactivated by 1-ethyl-3(3-dimethylaminopropyl)carbodi-imide and by phenylglyoxal, which implicates carboxylate and arginine residues respectively in the active site. We conclude that the β -galactosidase precursor is functionally identical to the mature lysosomal form of the enzyme and serves as an excellent enzyme source for investigation of structure–function relationships in the protein.

INTRODUCTION

The major form of lysosomal β -galactosidase (EC 3.2.1.23), a 64 kDa polypeptide, was first purified from human liver in 1974 by Norden et al. (1974). Now, a minor form (~800 kDa) is known to represent a complex composed of three polypeptides in addition to β -galactosidase; two of these are contributed by the protective protein, a heterodimer consisting of 32 and 20 kDa polypeptides derived by internal cleavage in the lysosome of a 52 kDa precursor. These are held together in the native subunit by interchain disulphide bonds (Verheijen et al., 1982). The third protein is neuraminidase (61 kDa). In human placenta, the complexed form of β -galactosidase represents ~15–20% of the total activity (Hubbes et al., 1992), but the bulk (~80–85%) of the enzyme occurs as a 134 kDa dimer (Cheetham, 1977; Norden et al., 1974; Jones et al., 1984). Pshezhetsky et al. (1992) have recently shown that the lysosomal pH and the membrane environment facilitate complex formation and stabilize enhanced β -galactosidase activity. That the complex is a real physiological entity was demonstrated conclusively by Galjaard, D'Azzo and colleagues (D'Azzo et al., 1982; Verheijen et al., 1982; Hoogeveen et al., 1983; Verheijen et al., 1985; Van der Horst et al., 1989), who characterized galactosialidosis, a disease in which deficiencies in both β -galactosidase and neuraminidase could be corrected by the action of the 'protective protein'.

Most procedures for the purification of β -galactosidase have employed tissue sources such as liver or placenta where both

complexed and non-complexed forms of the enzyme are isolated together. Purification of β -galactosidase to apparent homogeneity from these sources requires additional fractionation procedures, such as ion-exchange chromatography, which yields only 1–2% of the starting levels. Such preparations contain only the mature processed 64 kDa form of the enzyme. In the present paper we describe the characterization of the β -galactosidase precursor purified to apparent homogeneity from medium above permanently transfected Chinese hamster ovary (CHO) cells.

EXPERIMENTAL PROCEDURES

Cell culture

CHO cells, obtained from the American Type Culture Collection (Rockville, MD, U.S.A.), were grown in 100 mm² tissue culture dishes in 10 ml of Eagle's minimal essential medium (α -MEM) supplemented with 10% (w/v) fetal calf serum. Cells were maintained at 37 °C in a humidified 5% CO₂ environment until they were 80–85% confluent. Human skin fibroblasts used in this study were from a normal patient [HSC (Hospital for Sick Children) strain no. 3585], an infantile case of GM1-gangliosidosis (GM1, Case 1, ATCC no. GM05652A), and an adult case of GM1-gangliosidosis (GM1, Case 2, HSC strain no. 3749). To monitor the uptake of the precursor into these cells, the regular medium was aspirated, and the monolayers were incubated for 72 h in a 'defined medium' containing α -MEM

Abbreviations used: α -MEM, Eagle's minimum essential medium; CHO, Chinese hamster ovary; 2,4-DNPGal, 2,4-dinitrophenyl- β -D-galactopyranoside; DTT, dithiothreitol; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; 2FDNPGal, 2,4-dinitrophenyl-2-deoxy-2-fluoro- β -D-galactopyranoside; MBHS, maleimidobenzoyl-N-hydroxysuccinimide; PT-Gal, p-aminophenylthio- β -D-galactopyranoside; RSV- β -gal, pRc/RSV- β -galactosidase.

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with human serum albumin (0.25 mg/ml) with or without the addition of purified β -galactosidase precursor (1500 nmol/h per ml of medium) and 10 mM of either mannose-6-phosphate or mannose-1-phosphate. For measurement of β -galactosidase activity, the cells were washed several times, scraped into PBS, and lysed by sonication. For histochemical staining with indolyl- β -D-galactoside, monolayers were fixed for 5 min with ice-cold formaldehyde-glutaraldehyde fixative, and incubated as described by Lojda (1970).

Constructs

The cDNA clone for β -galactosidase (gp8) (inserted into the *SalI* site of pGEM-3Z) was generously provided by Drs. Y. Suzuki and A. Oshima (Oshima et al., 1988). The construction of pRc/RSV- β -galactosidase (RSV- β -gal) was carried out as follows. The β -galactosidase cDNA was released from pGEM-3Z gp8 by digestion with *HindIII*, which cleaves in the multiple cloning site upstream of the 5' end and at position 2309 downstream from the terminating codon. The purified cDNA was subcloned into the *HindIII* site of the mammalian expression vector pRc/RSV (Invitrogen), by ligation with 1 unit of International Biotechnologies (New Haven, CT, U.S.A.) T_4 ligase per mg of DNA at room temperature for 4 h. The orientation was checked by digestion with *EcoRI*.

Transfection

Known amounts of RSV- β -gal [20–50 mg of DNA in 50 ml of α -MEM was mixed with 30–90 mg of Lipofectin reagent (BRL) in 100 ml of α -MEM] were transfected into CHO cells according to the method of Felgner et al. (1987). pRc/RSV contains the *neo* gene, the protein product of which interferes with the effect of the drug G418, which blocks protein synthesis in mammalian cells by halting ribosomal function. The assumption is made that CHO cells in which the construct has been properly integrated into the genome should be resistant to the action of the drug in the medium. The cell layer was incubated for 4–6 h at 37 °C in a humidified, 5% CO₂ environment. An equal volume of α -MEM with 10% fetal calf serum was then added and the cells were incubated overnight. The medium was then replaced with 10 ml of α -MEM containing 10% fetal calf serum, and incubation was continued for 36 h.

Cells were subcultured (1:6 dilution) and grown in α -MEM containing G418 at a final concentration of 400–600 mg/ml. Incubation was carried out for 10–14 days. The cell population that adhered to the plate was considered to have incorporated the *neo* gene. The cells in each plate were washed in PBS several times and stained for 15 min with 1 ml of 0.2% (w/v) Trypan Blue. Each clone was marked on the bottom of the dish, scraped into a small drop of medium and aspirated with a plastic 1 ml pipette. These clones were later suspended in 2 ml of medium in a 24-well multiwell dish. Incubation was carried out in the presence of G418 as for the parental cells. After 5 days, the growth medium was tested for the level of β -galactosidase activities. For further purification of the β -galactosidase, the cells were maintained at 30 °C in CHO-S-SFM (GIBCO-BRL, Gaithersburg, MD, U.S.A.), a low-protein, defined medium specifically designed for recombinant CHO cell lines. Medium was changed every 6 days.

Purification of the precursor β -galactosidase from CHO cell medium

A 3 ml substrate-affinity column of *p*-aminophenylthio- β -D-galactopyranoside (PT-Gal)-Sephacrose, prepared as previously

described (Hubbes et al., 1992), was pre-equilibrated with buffer D [20 mM sodium acetate, pH 4.3, containing 0.1 mM dithiothreitol (DTT) and 300 mM NaCl] in the cold. Medium (600–800 ml), frozen immediately upon collection, was thawed and acidified to pH 4.3 with dropwise addition of cold glacial acetic acid. After centrifugation, the clear supernatant was allowed to percolate freely through the column, which was washed with 200 ml of buffer D, or until the A_{280} reached that of the buffer. The eluant (10 ml of 10 mM phosphate, pH 7.0, 0.1 mM DTT, 1.0 M NaCl and 0.1 M γ -D-galactonolactone) was applied, and fractions (1 ml) were collected manually, concentrated and dialysed against PBS.

Analytical procedures and enzyme assays

Protein determination

Protein concentration was determined by using the BioRad reagent according to the method described by Bradford (1976), with BSA as standard.

β -Galactosidase activity

β -Galactosidase activity was measured in duplicate using 4-methylumbelliferyl- β -D-galactopyranoside in 50 mM citrate, 100 mM phosphate buffer, pH 4.3, and incubated at 37 °C for 30 min (D'Agrosa et al., 1992). 4-Methylumbelliferone (Eastman) was used as standard.

For estimation of kinetic parameters, duplicate or triplicate continuous assays in the above buffer were done using 2,4-dinitrophenyl- β -D-galactopyranoside (2,4-DNPGal) at nine substrate concentrations over the range 0.045–0.337 mM. The initial rate of reaction was determined at 37 °C by monitoring the increase in A_{400} with a Pye Unicam PU8800 spectrophotometer equipped with a circulating water bath. The molar extinction coefficient (ϵ) for 2,4-dinitrophenolate was $7.15 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 4.3 and 37 °C.

Digestion with N-glycanase and endoglucosidase F

To 6 ml of precursor β -galactosidase (4 mg) was added 0.8 ml of buffer A (200 mM phosphate, pH 7.4, 100 mM EDTA, 10% v/v β -mercaptoethanol and 10% w/v SDS) in a final volume of 8 ml. After incubation for 30 min at 60 °C, 32 ml of buffer B (20 mM phosphate, pH 7.4, 10 mM EDTA, 1% β -mercaptoethanol, 1% v/v Triton X-100), and 0.08 unit of peptide-N-glycosidase or endoglucosidase F (Boehringer-Mannheim) were added (1 unit is 1 nmol of glycopeptide hydrolysed/min at pH 7.4). Digestion was continued overnight at 30 °C, and products were resolved on SDS/PAGE gels and stained with Coomassie Blue.

Molecular-sieve chromatography

The molecular mass of the enzyme was determined on a Sephacryl S-200 column (73 cm $\times$ 1.4 cm) monitored by measuring the A_{280} and, in the case of β -galactosidase, by also measuring the enzyme activity as described above. Elution volume was determined by weighing each collection tube before and after filling. Calibration standards included thyroglobulin (M_r 670 000), catalase (232 000), aldolase (158 000), BSA (67 000), ovalbumin (45 000) and ribonuclease (12 500). The precursor (0.5 mg) and each standard (0.5 mg) was applied in a sample volume of 0.5 ml.

Determination of the stereochemical course of hydrolysis by ¹H-n.m.r.

Solutions of 2,4-DNPGal and buffer materials were freeze-dried

twice from $^2\text{H}_2\text{O}$ to minimize ^1H -signals from the solvent. The enzyme was transferred to buffer containing $^2\text{H}_2\text{O}$ by ultra-filtration using 10 kDa nominal cut-off centrifugal concentrators (Amicon Corp., Danvers, MA, U.S.A.). ^1H -n.m.r. spectra were recorded on a Varian Unity 500 MHz spectrometer at 30 °C using a 5 mm sample tube. The reaction mixture, in a total volume of 0.6 ml, contained 1.8 mM 2,4-DNPGal, 50 mM citrate and 100 mM phosphate, pH 4.3, in $^1\text{H}_2\text{O}$. After acquisition of the initial spectrum, enzyme was added in sufficient quantity to complete hydrolysis in 1 h. The stereochemical course of the reaction was monitored by collecting spectra every 2 min for 1 h with a final spectrum at 5 h. Acquisition time was 1.36 s. Residual HO^2H was suppressed by weak presaturation during the 2 s recycle delay.

Effect of 2,4-dinitrophenyl-2-deoxy-2-fluoro- β -D-galactoside, carbodi-imide and phenylglyoxal

The purified precursor was inactivated with 2,4-dinitrophenyl-2-deoxy-2-fluoro- β -D-galactoside (2FDNPGal). Samples of 2FDNPGal at the concentrations indicated (six concentrations, from 0.0109 to 0.316 mM) were incubated in pH 4.3 citrate-phosphate buffer at 37 °C. Enzyme was added to each sample and aliquots (10 ml) were removed at intervals and diluted into 0.6 ml of 2,4-DNPGal substrate solution at the same pH to determine the residual activity at each time interval, by measuring the rate of 2,4-dinitrophenol release. The rate constant (k_{obs}) at each inactivator concentration was determined by fitting the residual activity versus time relationship to a first-order decay equation. K_i , the dissociation constant, and k_i , the rate constant for inactivation, were found by fitting the observed rate constants to:

$$k_{\text{obs}} = k_i[I]/(K_i + [I])$$

where $[I]$ is the inhibitor concentration, using a non-linear regression program (Leatherbarrow, 1990).

To assess the involvement of acidic amino acid residues in catalysis, the enzyme (13 μg /0.1 ml of final volume) was diluted into 100 mM sodium acetate buffer at pH 5.0 alone, or into the buffer containing 20 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide (EDC), or into the buffer containing both 20 mM EDC and 100 mM γ -D-galactonolactone. Incubations were continued for up to 150 min at room temperature. Aliquots were withdrawn at the indicated times, diluted 10-fold into assay buffer containing BSA (1 mg/ml), and assayed in triplicate. Similarly, to determine whether basic residues such as arginine were involved in the binding site of the enzyme, protein was diluted (13 mg/0.1 ml of final volume) into ice-cold 100 mM pyrophosphate at pH 8.3 alone, or into the pyrophosphate buffer containing 20 mM phenylglyoxal. Aliquots of the treated enzyme were diluted and assayed as mentioned above. This treatment was carried out at 4 °C because the enzyme was not stable at room temperature at this pH.

SDS/PAGE and Western blotting

For SDS/PAGE, samples were denatured in sample buffer (50 mM Tris/HCl, pH 6.8, 2% SDS, 0.1% v/v Bromophenol Blue, 10% v/v glycerol, 20 mM DTT) by incubating at 60 °C for 30 min. The following pre-stained molecular-mass standards (BRL) were used: myosin (M_r 215 500), phosphorylase *b* (105 100), BSA (69 800), ovalbumin (43 300), carbonic anhydrase (28 300), β -lactoglobulin (18 100) and lysozyme (15 400). Gels were stained using Coomassie Brilliant Blue R250.

For Western blotting, proteins were transferred to nitrocellulose in buffer (25 mM 3-[cyclohexylamino]-1-propane-

sulphonic acid, 20% v/v methanol, pH 10.0), and membranes were blocked (5% w/v skimmed milk, 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20). The nitrocellulose was incubated overnight at room temperature with 400 ng of affinity-purified anti-peptide IgG or with polyclonal anti-(placental β -galactosidase) IgG (D'Agrosa et al., 1992) in 10 ml of 1% Blotto (1% skimmed milk, 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), washed with 1% Blotto for 2 h, incubated for 1 h in 1:10000-diluted peroxidase-conjugated goat anti-(rabbit IgG) (Sigma) with 1% Blotto, and washed for 1 h in 1% skimmed milk. Reaction was detected after 5–20 min by the ECL-Hyperfilm-chemiluminescence method (Amersham International).

Affinity-purified anti-peptide antibody against the C-terminal end of β -galactosidase

The C-terminal β -galactosidase oligopeptide, (C)PPQKNKD-SWLDHV, was synthesized with a free carboxyl group on the terminal valine and a cysteine residue at the N-terminal to assist coupling reactions. To 10 mg of ovalbumin (Sigma) in 0.5 ml 100 mM phosphate buffer, pH 6.8, was added 0.05 ml of maleimidobenzoyl-*N*-hydroxysuccinimide (MBHS) dissolved in dimethylformamide to give a molar ratio of ovalbumin/MBHS of 1:27. After gentle stirring at room temperature for 30 min, the activated ovalbumin was eluted (0.5 ml) in the void volume of a 6 ml Sephadex G-25 column. This was added to 15 ml of a 1 mg/ml (10 nmol) solution of the oligopeptide dissolved in 20 mM EDTA, 100 mM phosphate buffer, pH 6.8, stirred for 4 h at room temperature, dialysed overnight in the cold against PBS, and then against water. The retentate was lyophilized. The ovalbumin-oligopeptide conjugate migrated with an M_r of 55 000 on SDS/PAGE gels, consistent with the coupling of 7 mol of peptide/mol of ovalbumin. The conjugate (4 mg) was injected into rabbits six times at 3 week intervals and the serum was collected. An oligopeptide-affinity column was synthesized by incubating CNBr-activated Sepharose 4B with 25 mg of oligopeptide dissolved in coupling buffer (Pharmacia). Purified IgG was applied to the oligopeptide-Sepharose 4B affinity column equilibrated in PBS and the unbound fraction was re-applied an additional eight times. The column was then washed with PBS and the bound IgG was eluted with 1 M glycine, 0.5 M KCl, pH 2.5. The protein in the peak tubes was adjusted to pH 7.0 with saturated Na_2CO_3 , dialysed overnight against PBS and used for Western-blot analysis. The yield of affinity-purified IgG was 0.03 mg/mg of crude IgG. Some properties of this anti-peptide antibody have been described previously (Hinek et al., 1993).

RESULTS

CHO clones expressing β -galactosidase

Sixteen clones resistant to G418 at 600 mg/ml medium were isolated, of which seven showed a 2–3-fold elevation of activity (20.5–29.3 nmol/h per ml of medium) over untransfected controls (10.3), four were in the 3–5-fold range (30.2–47.2), three were in the 8–10-fold range (87.2–98.0), and one was 12-fold elevated (124.4 ± 1.7), while only one clone (clone 38) showed a 19-fold enhancement (187.1 ± 0.9). The optimum incubation temperature to maximize production of the enzyme was 30 °C and the highest activity level in the medium was obtained after 7 days. This time was chosen for medium changes.

Structural characterization of precursor β -galactosidase

Precursor β -galactosidase was purified from the culture medium

Figure 1 Purification and immunodetection of the β -galactosidase precursor

The precursor (arrowhead, 88 kDa) purified from CHO cell medium by affinity column chromatography was subjected to SDS/PAGE electrophoresis as described. Lanes 2–5 (stained with Coomassie Blue) show the purified precursor loaded at 0.5, 1.0, 2.0 and 5.0 mg of protein respectively. Lane 6 shows the precursor (5 mg of protein) reacted strongly with the polyclonal anti- β -galactosidase antibody described by D'Agrosa et al. (1992). The band at 64 kDa which is clearly visible in lanes 4–6 is the processed, mature 64 kDa β -galactosidase polypeptide, and its occurrence reflects the extent of lysis of the host CHO cells. Lane 7 shows the strong reaction of the precursor (0.1 mg) with the affinity-purified C-terminal antipeptide antibody, whereas Lane 1 shows the Gibco-BRL pre-stained protein standards (phosphorylase *b*, M_r 105100; BSA, 69800; ovalbumin, 43300; carbonic anhydrase, 28300; trypsin inhibitor, 18100; and lysozyme, 15400).

by affinity chromatography on PT-Gal-Sepharose in a single step with a 279 ± 71 -fold (mean $\pm$ S.E.M., $n = 3$) enrichment and with an average overall yield of 42%. The final specific activity was 827 ± 368 nmol/h per mg of protein, comparable with results with the enzyme from human placenta, whereas the yield was 20-fold higher than that obtained from the placenta (Hubbes et al., 1992). The final preparation was homogeneous, as judged by SDS/PAGE and Coomassie Blue staining (Figure 1). As expected, the most abundant protein was the 88 kDa precursor. When the sample was deliberately overloaded to detect low-abundance proteins in the preparation, the material seen in the area of 64 kDa was estimated by densitometric analysis to be no more than 5% of the total (results not shown). The precursor

Figure 3 Digestion of precursor with N-glycanase

Equal samples of precursor (2 mg of protein) were digested at 30 °C with peptide-N-glycosidase F (80 m-units). Samples were as follows: undigested control (lane 2), digestion for 20 min (lane 3), 40 min (lane 4), 90 min (lane 5), 150 min (lane 6), and 24 h (lane 7). The products were analysed by SDS/PAGE on a 7% (w/v) polyacrylamide gel. Lane 1 shows the molecular-mass protein standards.

showed a strong reaction with the polyclonal antibody raised against the purified human placental enzyme (D'Agrosa et al., 1992) and the anti-(C-terminal peptide) antibody on Western blots (Figure 1). In direct immunoprecipitation experiments, the C-terminal antibody immunoprecipitated the enzyme activity in a dose-dependent manner (results not shown).

To determine the apparent molecular mass of the active precursor, the enzyme was chromatographed on a Sephacryl S-200 molecular-sieve column and eluted after the aldolase standard (Figure 2). Both the activity and the protein elution (A_{280}) profiles were coincident and the calculated apparent M_r from the standard curve was 105 500, indicating that the precursor is a monomer at physiological pH.

Prolonged digestion of the precursor with either endoglucosidase F or glycopeptidase F deglycosylated the precursor with a shift from ~ 88 kDa to ~ 72 kDa. When endoglucosidase F digestion was monitored over the course of 2.5 h, several (5–6) intermediates representing partially deglycosylated forms of the enzyme were clearly visible (Figure 3), suggesting that five of the

Figure 2 Molecular-sieve chromatography of β -galactosidase precursor

(a) The Sephacryl S-200 column was equilibrated in 10 mM sodium phosphate buffer, pH 7.4, containing 0.5 M NaCl. The column was monitored by measuring the A_{280} and also by measuring the β -galactosidase activity as described above. Elution volume was determined by weighing each collection tube before and after filling. (b) Standards shown include catalase (M_r 232 000), aldolase (158 000), ovalbumin (45 000) and ribonuclease (12 500). The precursor (0.5 mg; arrow) and each standard (0.5 mg) were applied separately in a sample volume of 0.5 ml.

Figure 4 Incorporation of precursor β -galactosidase into GM1-fibroblasts is blocked by mannose-6-phosphate

Normal fibroblasts show strong β -galactosidase histochemical reaction with indolyl- β -D-galactoside as substrate (A), whereas GM1-gangliosidosis cells (Case 1) do not (B). GM1 cells readily incorporate precursor from the medium and the histochemical reaction becomes indistinguishable from normal cells (C). Incorporation of the precursor is blocked by the presence in the medium of 10 mM mannose-6-phosphate (D).

seven putative N-glycosylation sites are likely to be occupied. In addition, the precursor, when added to the medium above GM1-gangliosidosis fibroblasts, was readily incorporated into the deficient cells, was localized to the lysosomal compartment (Figure 4) and normalized the enzyme activity (Table 1). Incorporation was inhibited by mannose-6-phosphate but was

unaffected by mannose-1-phosphate. These data indicate the monomeric precursor contains mannose-6-phosphate in some of its oligosaccharides (Cantor et al., 1992).

Kinetic characterization of the precursor

The purified precursor and the activity detected in the medium displayed a broad range of pH dependence, with 90 % of the activity spread over the pH range 3.4–5.5. Above pH 5.5, a small amount of activity was detectable up to $\sim$ pH 6.6, results similar to the placental enzyme (results not shown). An Eadie–Hofstee plot of enzyme activity determined with 4-methylumbelliferyl- β -D-galactoside at varying substrate concentrations gave a K_m of 0.29 mM and a V_{max} of 989 mmol/h per mg of protein, whereas with 2,4-dinitrophenyl- β -D-galactoside, a K_m of 0.73 mM and a V_{max} of 888 mmol/h per mg of protein were obtained, values in accord with the specific activities cited above.

$^1\text{H-n.m.r.}$ spectroscopy was used to determine whether the glycosidic bond is cleaved with retention or inversion of anomeric configuration, as shown in Figure 5 (Withers et al., 1986). Spectrum A (Figure 5) shows the anomeric proton region of the $^1\text{H-n.m.r.}$ spectrum of 2,4-DNPGal in $^1\text{H}_2\text{O}$. The doublet at 5.38 p.p.m. ($J = 7.8$ Hz) is due to the axial anomeric proton of the β -glycoside substrate, and the large resonance at 4.75 p.p.m. is largely from HO^1H . Spectra B and C were recorded at intervals after the addition of enzyme and clearly reveal first the appearance of a resonance at 4.59 p.p.m. due to the axial anomeric proton of β -D-galactose ($J = 7.9$ Hz) followed by a new resonance at 5.27 p.p.m. ($J = 3.2$ Hz) from α -D-galactose formed by mutarotation of the β -anomer. After 5 h (spectrum D), the substrate has been consumed and establishment of the equilibrium anomeric ratio ($\sim 2\beta/1\alpha$) indicates that the enzyme catalyses hydrolysis with net retention of its β anomeric configuration.

Kinetic parameters for inactivation by 2FDNPGal were determined by measuring inactivation rates at several different inhibitor concentrations as shown in Figure 6. Rapid irreversible inactivation was observed with kinetic parameters $k_i = 2.2 \text{ min}^{-1}$ and $K_i = 0.17 \text{ mM}$. Protection against inactivation was afforded by isopropyl β -D-thiogalactoside (5 mM), reducing the k_{obs} for inactivation at 0.079 mM 2FDNPGal from 0.67 to 0.38 min^{-1} . To further implicate acidic amino acid residues in the active site we attempted to inactivate the enzyme with a general group specific reagent, EDC, at pH 6.0 and at pH 4.3 in the presence or absence of γ -D-galactonolactone. The enzyme was rapidly inactivated to 50 % of the initial activity at pH 6.0, showed little inactivation at pH 4.3, and inactivation at pH 6.0 was blocked as expected by the addition of γ -D-galactonolactone, a competitive

Table 1 Correction of enzyme deficiency by uptake of precursor β -galactosidase

Monolayer cultures of GM1-gangliosidosis fibroblasts were incubated for 72 h in 10 ml of 'defined medium' [α -MEM with human serum albumin (0.25 mg/ml)] with or without the addition to the medium of purified β -galactosidase precursor (activity 1500 nmol/h per ml of medium) and 10 mM of either mannose-6-phosphate or mannose-1-phosphate. At the end of this time, the cells were washed several times and scraped into PBS, lysed by sonication and the activities in the cells were then measured (mean $\pm$ S.D., $n = 3$).

Treatment	Cell line ...	Activity (nmol/h per mg of protein)		
		Normal	GM1, case 1	GM1, case 2
Before incubation		788 $\pm$ 192	0.68 $\pm$ 1.1	17.7 $\pm$ 10.3
After 72 h incubation in the presence of:				
Defined media, no precursor		1067 $\pm$ 125	0.77 $\pm$ 1.3	19.6 $\pm$ 3.2
+ Precursor alone		2390 $\pm$ 645	1133 $\pm$ 115	1907 $\pm$ 618
+ Precursor + mannose-6-phosphate		895 $\pm$ 50	0.10 $\pm$ 0.14	20.6 $\pm$ 6.3
+ Precursor + mannose-1-phosphate		1613 $\pm$ 194	760 $\pm$ 165	694 $\pm$ 52

Figure 5 ^1H -N.m.r. spectra showing the stereochemical course of hydrolysis of 2,4-DNPGal by β -galactosidase

Spectra are of the anomeric proton region of the substrate and product before addition of enzyme (A) and at 25, 55 and 300 min after addition (B, C, and D respectively). For details, see the Experimental procedures section.

Figure 6 Inactivation of β -galactosidase by 2FDNPGal

(a) β -Galactosidase was assayed with 2,4-DNPGal following incubation with 0.011 mM ($\blacktriangle$), 0.025 mM ($\triangle$), 0.049 mM ($\blacksquare$), 0.079 mM ($\square$), 0.16 mM ($\bullet$), and 0.32 mM ($\circ$) 2FDNPGal for the times indicated. Lines represent first-order fits to the observed data. (b) Replot of pseudo-first-order rate constants determined from (a) versus concentration of 2FDNPGal. The line represents fit to the equation described in the Experimental procedures section.

inhibitor of the enzyme (results not shown). We next examined the effect of phenylglyoxal on the activity of the enzyme at pH 8.3. This reagent has been used to implicate arginine residues in the active sites of enzymes, such as human hexosaminidase (Brown and Mahuran, 1991). At pH 8.3, the β -galactosidase precursor was unstable to incubation at room temperature, so

Figure 7 Inactivation of β -galactosidase by phenylglyoxal

The reactions were carried out as indicated in the Experimental procedures section. $\circ$, The enzyme in buffer alone; $\square$, the non-reversible inhibitory effect of 20 mM phenylglyoxal.

the effect of phenylglyoxal was assessed at 4 $^{\circ}\text{C}$, where the activity was stable over the duration of the experiment (Figure 7). The enzyme was rapidly inactivated by treatment with 20 mM phenylglyoxal. It was impossible to evaluate the protective effect of γ -D-galactonolactone under these conditions as the inhibitor is not expected to bind to the enzyme at this pH.

DISCUSSION

Most procedures for the purification of β -galactosidase have employed tissue sources such as liver or placenta where both complexed and non-complexed forms of the enzyme are isolated together. Purification of β -galactosidase to apparent homogeneity from these sources requires many fractionation procedures with yields of only 1–2% of the starting levels. Such preparations contain only the mature, processed 64 kDa form of the enzyme. In the present work we describe the characterization of the β -

galactosidase precursor purified to apparent homogeneity from medium above permanently transfected CHO cells. We have demonstrated that purified precursor β -galactosidase is enzymically active with the same pH optimum, and K_m values as the mature form of the enzyme, and the precursor corrects the β -galactosidase deficiency in cultured GM1-gangliosidosis fibroblasts. The purified precursor migrates on SDS/PAGE gels, even at low concentrations, as a relatively wide band corresponding to an M_r range of 84000–88000, suggesting that the precursor is polydisperse probably due to variations in the extent of glycosylation, i.e. not every molecule of the enzyme has exactly the same number and/or level of processing of their attached oligosaccharide chains. The results of monitoring the deglycosylation to the 72 kDa apoprotein with timed endoglycosidase digestions suggest the presence of five oligosaccharide chains, consistent with the deduced sequence which shows the occurrence of seven possible N-glycosylation sites. However, Yamamoto et al. (1990) found during direct peptide sequencing that a putative N-glycosylation site (N464) was not occupied. Furthermore, the deduced sequence from residues 542–547 contains two side-by-side consensus sequences (N⁵⁴²SS and N⁵⁴⁶YT), and steric hindrance makes it unlikely that oligosaccharides reside side-by-side on both asparagines, thus reducing the probable number of oligosaccharides to five, consistent with our findings.

As the major polypeptide chain of the deglycosylated precursor is 72 kDa and that of the deglycosylated mature lysosomal enzyme is 59 kDa (D'Agrosa et al., 1992), post-translational processing must result in the loss of ~ 13 kDa from the precursor apoprotein. Because the C-terminal anti-peptide antibody reacts strongly with the 88 kDa precursor, whereas the mature glycosylated 64 kDa form is not detected, we consider this direct evidence that post-translational processing involves at least some proteolytic cleavage at the C-terminal end of the protein. There have been no reports directly identifying the C-terminal residue of the mature processed polypeptide and the nature and extent of this processing remains to be defined. However, Yamamoto et al. (1990) sequenced a tryptic peptide from the liver enzyme with F⁴⁹² as the last detectable residue and, based on the deduced protein sequence, suggested that the hydrophilic sequence close to residue R⁵³⁰ might constitute the C-terminal end of the mature protein. Recent analyses of mutations in the β -galactosidase gene (Yoshida et al., 1991, 1992) have identified at least six mutations in exons 15 and 16, which encode this C-terminal region (residues 494–677) of the protein, and within the putative area that is subject to proteolytic cleavage during processing to the mature enzyme. These mutations have been described largely in patients with the most clinically severe forms of β -galactosidase deficiency. The impact that these mutations have on the functioning of the protein is either to affect the terminal lysosomal processing of the precursor so that the mature form of the enzyme is rapidly degraded in the lysosome, or to alter the folding and conformation of the precursor resulting in rapid turnover in the endoplasmic reticulum. In either case, the net result would be decreased amounts of the mature enzyme, predictions in accord with earlier studies (D'Agrosa et al., 1992).

The fact that β -galactosidase can be isolated as a fully functional, stable precursor without the need for the co-expression of the protective protein implies that its secretion, i.e. trafficking from the endoplasmic reticulum through the Golgi complex, from the CHO cells does not require the formation of any part of the β -galactosidase–neuraminidase–protective protein complex. Furthermore, no other members of the complex are needed in order for β -galactosidase to fold into an active state, which is presumed to happen soon after its synthesis. The fact

that it, like other expressed proteins, is secreted from the CHO cells probably reflects saturation of the phosphomannose receptor, which has previously been shown to display Michaelis–Menten saturation kinetics (Kornfeld, 1992). The occurrence of the precursor as a monomer, coupled with the recent data of Pshezhetsky et al. (1992) and Pshezhetsky and Potier (1993), indicates that the dimerization and oligomerization of the enzyme and its association with protective protein and neuraminidase to form the complex is largely an endosomal–lysosomal event that occurs after acidification within this compartment.

The stereochemical course of enzymic hydrolysis was shown unequivocally to proceed with retention of configuration at the anomeric center of the sugar. Generation of a covalent intermediate equivalent to that involved in this 'retaining' mechanism was achieved with the mechanism-based inactivator 2FDNPGal, resulting in rapid inactivation of the enzyme. Inactivation by this reagent and by EDC, and protection against inactivation by isopropyl β -D-galactoside or by γ -D-galactonolactone, suggest the direct involvement of a carboxylic acid group of an acidic amino acid in the active site, where it functions in catalysis as the normal enzymic nucleophile (Sinnott, 1990; Gebler et al., 1992; Kempton and Withers, 1992). Gebler et al. (1992) have demonstrated unequivocally the efficacy of this approach in the case of purified *Escherichia coli* β -galactosidase by direct isolation and amino acid sequencing of the fluoro-galactose-containing peptides.

Finally, the concept of the β -galactosidase–protective protein–neuraminidase complex has so far focused on its role in lysosomal digestion of macromolecules (Pshezhetsky and Potier, 1993). However, recent evidence suggests that β -galactosidase when secreted may have a functional role in the hydrolysis of glycoprotein oligosaccharides at neutral pH values (Skudlarek et al., 1992) and may function along with sialidase at the cell surface to help convert latent cytokines into fully functional ones (Yamamoto and Kumashiro, 1993). This is of great interest in view of the recent observations of Jackman et al. (1992) that the protective protein when secreted from platelets functions as a deamidase. Our recent results have shown that a form of β -galactosidase, the so-called alternately spliced Gal-like variant, is immunologically related to the 67 kDa elastin-binding protein of the cell surface elastin-binding receptor (Hinek et al., 1993), which also contains a 61 kDa protein and a 55 kDa protein, the identities of which are unknown (Hinek et al., 1988). Thus secreted β -galactosidase may have important heretofore unknown actions at cell surfaces.

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