Biochemical properties of recombinant human β -glucuronidase synthesized in baby hamster kidney cells

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The cDNA sequence encoding human β -glucuronidase [Oshima, Kyle, Miller, Hoffmann, Powell, Grubb, Sly, Troplak, Guise and Gravel (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 685–689] was expressed in baby hamster kidney (BHK) cells. After purification from the culture supernatant in one step by use of immunoaffinity chromatography, the biochemical properties of the enzyme were examined. With a pH optimum of 4.0, a K_m of 1.3 mM and thermal stability up to 68 °C, this protein has characteristics very similar to those described for β -glucuronidase from human placenta [Brot, Bell and Sly (1978) *Biochemistry* **17**, 385–391]. However, the recombinant product has several structural properties not previously reported for β -glucuronidase isolated from natural sources. First, recombinant β -glucuronidase is synthesized as a tetramer consisting of two disulphide-linked dimers. As can be inferred from the cDNA sequence, the enzyme possesses five cysteine residues after cleavage of the signal peptide. By introducing a C-terminal truncation, we eliminated the last cysteine at position 644. In the mutant, covalent linkage between two monomers is no longer observed, indicating that Cys-644 is involved in intermolecular disulphide-bond formation. The functional role of the disulphide bond remains elusive, as it was shown that (i) intracellular transport of the mutant is not

impaired and (ii) it is still able to form an enzymically active tetramer. A second feature that has not previously been observed for β -glucuronidase from any origin is the existence of two enzymically active species for recombinant β -glucuronidase, when examined by gel filtration on a TSK 3000 column. With apparent molecular masses of 380 kDa and 190 kDa we propose that they represent tetramers and dimers respectively. Partial N-terminal sequencing and electrophoresis under denaturing conditions revealed that the dimers consist of subunits that have been proteolytically processed at their C-terminus losing 3–4 kDa in peptide mass. Controlled proteolysis demonstrates that the enzyme's overall protein backbone as well as its activity are resistant to a number of proteases. Only the C-terminal portion is susceptible to protease action, and the disulphide-linked form is readily converted into non-disulphide-bonded subunits. Pulse-chase analysis shows that human β -glucuronidase remaining intracellular in BHK cells after synthesis undergoes a similar proteolytic processing event, i.e. a reduction in mass of 3–4 kDa. Like purified and proteolytically processed β -glucuronidase, the intracellular form behaves as a dimer on gel filtration, indicating that the processing event in the cell leads to a different oligomeric structure of the enzyme.

INTRODUCTION

Human β -glucuronidase is a lysosomal enzyme responsible for O-glycosyl bond hydrolysis. Among its natural substrates in men are dermatan sulphate, chondroitin sulphate and heparan sulphate. Enzyme deficiency leads to a disease known as mucopolysaccharidosis type VII (MPSVII or Sly syndrome) which results from lysosomal storage of undegraded glycosaminoglycans in the spleen, liver, kidney, brain and skeletal system (Sly et al., 1973).

The catalytically active enzyme isolated from human placenta has been described as a tetramer composed of four identical subunits of 77 kDa (Brot et al., 1978). Each polypeptide chain is initially synthesized on membrane-bound ribosomes and translocated into the lumen of the endoplasmic reticulum during or immediately after synthesis. Within the endoplasmic reticulum, core glycosylation at all four potential glycosylation sites and assembly to a tetramer occur (Shipley et al., 1993a). When the enzyme is transported through the Golgi complex, the glycans are modified and high-mannose-type oligosaccharides receive a specific recognition marker, mannose 6-phosphate, which serves as a signal for the mannose 6-phosphate receptor (von Figura and Klein, 1979; Goldberg and Kornfeld, 1981; Sly and Fisher, 1982). This receptor directs lysosomal enzymes to a prelysosomal compartment where the receptor–enzyme complex dissociates at

low pH. While the receptor recycles back to the Golgi, the enzyme moves on for final packaging into lysosomes (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). In addition to the post-translational modifications that affect the carbohydrate moieties, β -glucuronidase molecules undergo proteolytic processing during their transit to the lysosomes. As has been shown for β -glucuronidase from mouse (Brown et al., 1981), rat (Rosenfeld et al., 1982), pig (Erickson and Blobel, 1983) and man (Oshima et al., 1987), newly synthesized β -glucuronidase is converted from a precursor form into a final mature form which is reduced by about 2–4 kDa in peptide mass. Further characterization revealed that the proteolytic event is likely to occur at a prelysosomal site (Gabel and Foster, 1987) and the peptide is cleaved from the C-terminus (Erickson and Blobel, 1983). In mice, the C-terminal propeptide is responsible for retention of β -glucuronidase in the endoplasmic reticulum by association with the resident protein egasyn (Medda and Swank, 1985; Medda et al., 1989). Egasyn, which is identical with mouse esterase-22, binds at its active site to the propeptide which has sequence similarity to the reactive site region of the serpin superfamily (Li et al., 1990). The complex-formation leads to a dual localization of murine β -glucuronidase, with activity associated with both lysosomes and endoplasmic reticulum (Medda et al., 1987), a phenomenon that has not been observed for human β -glucuronidase (Ovnic et al., 1991). Recently, the cleavage site for

the C-terminal propeptide of human placental β -glucuronidase has been determined to be the peptide bond between Thr-633 and Arg-634 (Islam et al., 1993).

The aim of the present study was to characterize the biochemical properties of human β -glucuronidase expressed in baby hamster kidney (BHK) cells. Additional information about the structure of the enzyme will facilitate further studies of cellular processing and will be needed when the recombinant protein is considered for enzyme-replacement therapy in patients with mucopolysaccharidosis type VII (Bougharios et al., 1993) or is used as part of a fusion protein in antibody-based tumour therapy (Bosslet et al., 1992, 1994). In the present paper we provide data on the subunit structure of recombinant β -glucuronidase showing that the secreted enzyme is a tetramer consisting of paired disulphide-bonded subunits. On proteolytic cleavage, resembling that observed for intracellular β -glucuronidase, the oligomeric structure is changed from a tetramer to a dimer. Furthermore, intracellular human β -glucuronidase in transfected BHK cells corresponds mainly to the processed form and behaves as a dimer on gel filtration.

MATERIALS AND METHODS

Plasmid construction

The plasmid pGEM4-HUGP 13 containing the full-length cDNA of human β -glucuronidase (Oshima et al., 1987) was used as a template for PCR following standard conditions (Orlandi et al., 1989). The oligonucleotide primer pair BWfor1 5'-AAATCTAGATCAAGTAAACGGGCTGTT-3' and BWback 5'-TTTAAGCTTATGGCCCGGGGTCGGCG-3' amplified a DNA fragment coding for the complete β -glucuronidase including the N-terminal signal sequence. Amplification of a 3'-end-truncated version of the enzyme was accomplished with the primer pair BWfor2 5'-AAAATCTAGATCA-GTGGGGATACCTGG-3' and BWback. In the resulting fragment nucleotides 3793–3835 were deleted and a stop codon introduced after the CAC/His-637 codon. The PCR primer brought in a *Hind*III restriction-enzyme site upstream of the translation initiation signal and a *Xba*I restriction-enzyme site downstream of the stop codon. After digestion with *Hind*III and *Xba*I the fragments were gel purified using a GeneClean II kit (Bio 101) and cloned into a similarly digested pABstop expression vector (Zettlmeissl et al., 1987) to produce pMCG-GLUC and pMCG-dGLUC.

DNA inserts were sequenced by the dideoxynucleotide-termination method (Sanger et al., 1977) using the U.S. Biochemical Corp. sequence kit, according to the manufacturer's manual.

Cell culture and transfection

BHK cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal calf serum. Transfection of BHK cells was performed using the calcium phosphate coprecipitation technique (Graham and van der Eb, 1973) with 5 μ g of plasmid pRMH 140 carrying a neomycin-resistance gene and 10 μ g of pMCG-GLUC or pMCG-pGLUC respectively. Selection medium containing 0.4 μ g/ml G418 was added 2 days later and, after 2 weeks, surviving clones were screened by the enzyme activity test for β -glucuronidase secretion. The positive clones B702 and B688 were expanded. For large-scale production of β -glucuronidase the cells were grown in selection medium in roller flasks. Just before confluency the medium was changed and the cells were grown for 2 days in

DMEM without fetal calf serum. Medium from several roller flasks was collected and subjected to immunoaffinity chromatography.

Immunoaffinity chromatography and h.p.l.c. analysis

Before concentrating the supernatant containing β -glucuronidase about 50-fold using an ultrasette tangential flow device with a 30 kDa membrane (Filtron Technical Corp.), the medium was passed through a 0.45 μ m membrane (Pall) followed by a 0.2 μ m membrane in order to remove any cells and cell debris. The concentrated and cleared supernatant was applied to an anti- β -glucuronidase affinity column (1 cm $\times$ 5 cm). Antibody-Sepharose was prepared by coupling Protein A-Sepharose-purified anti- β -glucuronidase monoclonal antibody 2118 to CNBr-activated Sepharose 4B (Pharmacia) at a concentration of 5 mg of antibody/ml of gel (van Eijk and van Noort, 1976). After the column had been washed with several column volumes of PBS, pH 7.2, elution was started with PBS, pH 5, at a flow rate of 20 ml/h. Fractions were collected every 3 min and the A_{280} was monitored. The fractions containing the bulk of enzyme activity were pooled and adjusted to pH 7.2 with NaOH. The pooled enzyme fraction was then either analysed directly by SDS/PAGE and immunoblotting or further concentrated with a Microsep 30000 (Filtron) for h.p.l.c. analysis. Gel filtration was performed on an h.p.l.c. apparatus from Merck-Hitachi equipped with an L-6210 pump, L-4000 u.v. detector and D-2500 chromato-integrator. Samples were applied to a TSK G3000-SWXL column (7.8 cm $\times$ 30 cm) equilibrated with PBS, pH 7.4. Samples from cell lysates were prepared by incubating 5×10^6 cells in Nonidet P40-containing lysis buffer [50 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$, 0.5% (v/v) Nonidet P40] for 30 min at 4°C. Nuclei and debris were removed by centrifugation at 13000 g for 5 min and the supernatant was passed through a 0.2 μ m filter (Schleicher and Schuell). The column was run at a flow rate of 0.5 ml/min and after 10 min five fractions/min were collected. The void volume was determined with Blue Dextran and the column was calibrated with carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), β -amylase (200 kDa) and apoferritin (443 kDa) from the MW-GF-1000 kit of Sigma.

Gel electrophoresis, immunoblotting and staining

The different forms of β -glucuronidase were analysed by electrophoresis on SDS/7.5% polyacrylamide gels (Laemmli, 1970). Samples were solubilized in SDS sample buffer [62.5 mM Tris/HCl, pH 6.8, 10% (v/v) glycerine, 4% (w/v) SDS, 0.1% Bromphenol Blue] in the presence or absence of 5% (v/v) 2-mercaptoethanol. Gels were either stained with a Proteicolor silver stain kit as described by the manufacturer (Roth) or dried for visualization of proteins by autoradiography. The electrophoretic transfer of proteins to nitrocellulose sheets was as described previously (Towbin et al., 1979). The filters were placed in blocking buffer [10 mM Tris/HCl, pH 7.5, 15 mM NaCl, 1% (v/v) skimmed milk powder and 1% (v/v) human serum albumin] for 1 h and incubated overnight with mouse anti-(human β -glucuronidase) monoclonal antibody 2156/215 (diluted 1:50 in blocking buffer), washed three times for 10 min each in TBS buffer (50 mM Tris/HCl, pH 7.4, 200 mM NaCl) and incubated for 1 h with peroxidase-labelled goat anti-mouse IgG (1:1000 in blocking buffer). After three additional washes with TBS buffer, peroxidase activity was detected using 4-chloro-1-naphthol as substrate and H_2O_2 as catalyst.

Proteolytic digestion of β -glucuronidase

Proteolysis with trypsin was carried out in 0.1 M Tris/HCl, pH 8, containing 0.02 M CaCl_2 , proteolysis with endoproteinase Lys-C was performed in 0.025 M Tris/HCl, pH 8, containing 1 mM EDTA and proteolysis with endoproteinase Arg-C in 0.1 M KH_2PO_4 , pH 8. All digestions were performed at 37 °C at a protease/substrate ratio of 1:10 or 1:1 for the incubation times noted.

Metabolic labelling and immunoprecipitation

Cells were grown to subconfluency in 25 cm² tissue culture flasks in DMEM with 10 % fetal calf serum. Before labelling, cells were incubated for 30 min in methionine-free DMEM with 10 % fetal calf serum. Each flask was labelled with 50 μCi of [³⁵S]methionine for 30 min at 37 °C. Incorporation of label was stopped by the addition of non-radioactive methionine to a final concentration of 1 mM and the cells were chased for 0, 0.5, 1, 3 and 8 h. The supernatant containing the secreted β -glucuronidase was collected and the cells were lysed in Nonidet P40 lysis buffer. The glycoprotein β -glucuronidase was recovered from the culture medium and lysates by immunoprecipitation using goat anti-(human β -glucuronidase) antibody and Protein G-Sepharose (Pharmacia). The immune complexes were washed five times with buffer [50 mM Tris/HCl, pH 7.4, 0.5 % (v/v) Nonidet P40, 5 mM EDTA, 150 mM NaCl], and stored either as dry pellets at -20 °C or analysed directly.

Digestion with protein N-glycosidase F

Purified β -glucuronidase was heated at 95 °C for 5 min in 10 mM Tris/HCl, pH 7, containing 1 % SDS and 5 % 2-mercaptoethanol. After centrifugation, samples of the supernatant were diluted 10-fold in 50 mM sodium phosphate, pH 7.6, containing 10 mM EDTA, 0.5 % Nonidet P40, 0.5 % 2-mercaptoethanol and 1 mM phenylmethanesulphonyl fluoride and digested with 0.5 unit of N-glycosidase F for 18 h at 37 °C.

N-Terminal sequencing

N-Terminal sequences were determined on an Applied Biosystems Automatic micro sequencing apparatus (model 477A).

Assays

β -Glucuronidase activity was assayed fluorimetrically. The reaction mixture consisted of 75 μl of 0.01 % BSA/200 mM sodium acetate, pH 5, containing 2.5 mM of 4-methylumbelliferyl β -glucuronide (Sigma) and 25 μl of the enzyme solution. After incubation at 37 °C for 10 min, 1.5 ml of 0.2 M glycine/0.2 % SDS, pH 11.7, was added to stop the reaction. One unit of enzyme activity is defined as the amount of enzyme hydrolysing 1 μmol of substrate/h under the assay conditions. Specific activity is expressed as units/mg of protein. The Bio-Rad Protein Assay (Bio-Rad Laboratories) with BSA as standard was used to determine protein.

K_m was determined under the above-mentioned assay conditions and was calculated with the software GraFit (Erithacus Software Ltd., Staines, Middx., U.K.). The pH optimum was determined with 4-methylumbelliferyl β -glucuronide in 200 mM sodium acetate/0.01 % BSA. Thermal stability at 68 °C was tested in the same buffer at pH 6.

RESULTS

Comparison of wild-type β -glucuronidase with C-terminal-truncated mutant

The expression vectors pMCG-GLUC and pMCG-dGLUC containing cDNA coding for human β -glucuronidase and a C-terminal-truncated form of β -glucuronidase respectively were transfected into BHK cells. The stable clones B702 expressing wild-type and B688 expressing mutant β -glucuronidase were selected for further studies. In the mutant enzyme a stop codon was introduced after the CAC/His-637 codon, thereby eliminating 14 amino acids from the C-terminus. The deletion starts at a site showing similarity to the reactive-site region of serpins where cleavage occurs and includes the last of five cysteines at position 644. Enzymes secreted into the supernatant were immunoprecipitated with goat anti-(human β -glucuronidase) serum and analysed by Western blotting. For wild-type β -glucuronidase a band of approximately 83 kDa is observed under reducing conditions and a band of 165 kDa under non-reducing conditions (Figure 1, lanes 1 and 3), indicating that it is a disulphide-bonded dimer. However, the mutant migrated, under reducing and non-reducing conditions, at the same position slightly below the 83 kDa monomer of the wild-type enzyme (Figure 1, lanes 2 and 4). The unchanged mobility of the mutant under reducing conditions demonstrates that it lacks the interchain disulphide bond present in the intact enzyme. The results suggest that Cys-644 is involved in disulphide-bond formation but allow no conclusion about the cysteine residue that Cys-644 is paired with.

Purification and properties of human β -glucuronidase

To analyse the enzyme in more detail we purified β -glucuronidase secreted by BHK cell clones B702 and B688. As it appears that the mode of purification may affect the properties of the enzyme, the steps involved in purification, as exemplified for B702, are presented in brief.

After 2 days of culture of B702 in roller bottles the medium contained about 20 units of β -glucuronidase/mg of protein. Any residual cells and cell debris in the spent medium were removed by filtration before concentrating it about 50-fold. The concentrated and cleared supernatant was applied to an anti- β -glucuronidase affinity column, prepared as described in the Materials and methods section. The column was extensively washed with PBS, pH 7.2, followed by an elution step at pH 5.

Figure 1 Secretion of wild-type and mutant β -glucuronidase by transfected BHK cells

Human β -glucuronidase was immunoprecipitated from the culture media using goat anti- β -glucuronidase serum and analysed by Western blotting after SDS/PAGE. The molecular masses (kDa) of standard proteins are indicated. Samples in lanes 1 and 2 contained 5 % 2-mercaptoethanol; those in lanes 3 and 4 contained no reducing agent. Lanes 1 and 3, wild-type β -glucuronidase; lanes 2 and 4, mutant β -glucuronidase.

Figure 2 SDS/PAGE analysis of immunoaffinity-purified wild-type and mutant β -glucuronidase

Silver-stained gel (7.5%) of wild-type (lanes 1 and 3) and mutant (lanes 2 and 4) β -glucuronidase. Samples in lanes 1 and 2 were reduced, samples in lanes 3 and 4 were not.

Figure 3 *N*-glycosidase F treatment of purified wild-type β -glucuronidase

β -Glucuronidase (50 μ g/ml) was incubated for 18 h at 37 °C in the absence (lane 1) or presence (lane 2) of 0.5 unit of protein *N*-glycosidase F/ml. Portions were subjected to SDS/PAGE followed by immunoblotting.

A single protein peak was eluted which corresponded to the enzyme activity (results not shown). The pooled enzyme fractions were analysed by SDS/PAGE directly or further concentrated for h.p.l.c. analysis. The specific activity of the purified enzyme was 1880 units/mg of protein. With a pH optimum of 4, a K_m of 1.3 mM and thermal stability at 68 °C for up to 3 h at pH 6 (results not shown), the properties are similar to those of β -glucuronidase isolated from human placenta (Brot et al., 1978). The B688 mutant enzyme was purified in the same way and had the same pH optimum, K_m value and thermal stability as the B702 wild-type.

In contrast with the single band for β -glucuronidase obtained on SDS/PAGE after immunoprecipitation and Western blotting directly from the supernatant, affinity-purified β -glucuronidase from B702 reveals three bands after SDS/PAGE followed by silver staining. In addition to the 83 kDa band, two faster-migrating species with an apparent molecular mass of 79 kDa and 64 kDa are observed in the presence of reductant (Figure 2, lane 1). All bands visible in the silver-stained gel reacted with mouse anti-(human β -glucuronidase) monoclonal antibody 2156/215 in immunoblot analysis (results not shown). As direct immunoprecipitation from the supernatant as well as pulse-chase

analysis (see below) revealed that the enzyme is initially secreted as a molecule with intact 83 kDa monomers, we conclude that the two faster-migrating species are proteolytic fragments of the enzyme. A further fragment of 18 kDa was observed in 13% polyacrylamide gels (results not shown). Partial proteolysis could be reduced but not completely blocked when the protease inhibitors aprotinin, leupeptin and phenylmethanesulphonyl fluoride were present during purification. Under non-reducing conditions, the 83 kDa monomer disappears and a band of 165 kDa representing a covalent dimer is observed (Figure 2, lane 3). However, the proteolytically derived fragments still migrate at the same position. These results suggest that the proteolytic event in human β -glucuronidase caused the loss of the intermolecular disulphide bond between two monomers present in the intact enzyme. For the purified mutant no fragments are observed, and the enzyme behaves as the proteolytic fragments in that no change in mobility is observed on SDS/PAGE under reducing compared with non-reducing conditions (Figure 2, lanes 2 and 4).

To determine whether the decrease in molecular mass of the 79 kDa fragment of purified wild-type β -glucuronidase is due to protein or carbohydrate processing, we digested the protein with protein *N*-glycosidase F. This enzyme cleaves the bond between asparagine and the first *N*-acetylglucosamine residue of *N*-linked oligosaccharide chains. Protein *N*-glycosidase F treatment of β -glucuronidase reduced the 83 kDa fragment by about 8 kDa and the 79 kDa fragment about 6 kDa (Figure 3). This result suggests that, although the difference in mass between the two fragments is largely due to protein processing, the 79 kDa fragment contains less carbohydrate than the 83 kDa precursor.

Controlled proteolysis of β -glucuronidase

To demonstrate that proteases can account for the loss of the intermolecular disulphide bridge between two β -glucuronidase monomers, the purified wild-type enzyme was subjected to limited proteolysis. Proteolysis experiments were carried out with endo-proteinase Arg-C, endoproteinase Lys-C and trypsin in appropriate buffer systems at 37 °C with a β -glucuronidase/protease ratio of 10:1. Arg-C, Lys-C and trypsin were chosen because they have an increasing number of potential cleavage sites in β -glucuronidase. Under reducing conditions, β -glucuronidase incubated in the absence of protease (Figure 4a, lanes 1, 4 and 7) generated on SDS/PAGE a pattern essentially identical with that generated by β -glucuronidase incubated in the presence of any of the three different proteases (Figure 4a, lanes 2, 5 and 8). The incubation mixture with protease only is shown in Figure 4(a) lanes 3, 6 and 9. We conclude that, despite the presence of protease, β -glucuronidase subunits remain largely intact and no cleavage to subfragments of appreciable size occurs. Under non-reducing conditions, β -glucuronidase incubated in the absence of protease shows the typical shift of the 83 kDa monomer to the 165 kDa dimer position, whereas, again, the faster-runner fragments did not change their position compared with reducing conditions (Figure 4b, lanes 1, 4 and 7). However, under the action of trypsin all dimers were converted into monomers. Treatment with Lys-C resulted in some cleavage and Arg-C caused no disappearance of the covalently linked dimer under the conditions used in this experiment (Figure 4b, lanes 2, 5 and 8). Samples taken from the reaction mixture and tested for activity showed that no activity was lost. Even after an overnight incubation at a substrate/protease ratio of 1:1 no inactivation of β -glucuronidase was observed (results not shown). Thus, although the overall protein backbone and enzyme activity were remarkably resistant to the action of the proteases used,

Figure 4 Controlled proteolysis of recombinant wild-type β -glucuronidase

Samples of 5 μ g of β -glucuronidase were incubated at 37 °C for 1 h in either in presence of trypsin (lane 2), endoproteinase Lys-C (lane 5) or endoproteinase Arg-C (lane 8). Undigested control samples of β -glucuronidase in the respective incubation buffer are shown in lanes 1, 4 and 7. Protease only is shown in lane 3 for trypsin, lane 6 for Lys-C and lane 9 for Arg-C. All enzymes were used at a ratio of protease/ β -glucuronidase of 1:10 (w/v). Samples were analysed by SDS/PAGE under reducing conditions (a) or non-reducing conditions (b) followed by silver staining.

Figure 5 Molecular-mass estimations of native wild-type and mutant β -glucuronidase

The molecular mass of the purified proteins was determined by h.p.l.c. analysis on a calibrated TSK G3000 column. Conditions and standard proteins are described in the text. $\circ$, B702 (T), wild-type β -glucuronidase tetramer; $\square$, B702 (D), wild-type β -glucuronidase dimer; $\triangle$, B688, mutant β -glucuronidase.

trypsin and Lys C were able to cleave the covalent linkage between the β -glucuronidase dimers. Our observation extends earlier work showing that trypsin treatment of microsomal rat liver β -glucuronidase converts its electrophoretic and isoelectric focusing properties into those resembling lysosomal β -glucuronidase (Owens et al., 1975; Owens and Stahl, 1976).

Oligomeric structure of native β -glucuronidase

Native β -glucuronidase is assumed to be a tetramer of molecular mass 332 kDa (Drendel et al., 1993). To test whether the intermolecular disulphide bond is involved in oligomerization of subunits, h.p.l.c. of immunoaffinity-purified material was performed. The apparent molecular mass of β -glucuronidase was

Figure 6 SDS/PAGE analysis of the tetrameric and dimeric form of β -glucuronidase

Purified wild-type β -glucuronidase was subjected to h.p.l.c. A portion of each fraction containing β -glucuronidase eluted as a tetramer and dimer were analysed by SDS/PAGE. In the silver-stained gel, lanes 1 and 3 show subunits of the β -glucuronidase tetramer and lanes 2 and 4 subunits of the dimer. Samples in lanes 1 and 2 were reduced, samples in lanes 3 and 4 were not.

determined with a TSK G3000 column at pH 7.4 by comparing the ratio of V_e/V_o for the purified protein with that of protein standards of known molecular mass (V_e is the elution volume and V_o is the void volume). Mutant β -glucuronidase was eluted as a single peak with an apparent mass of 380 kDa, indicating that it is a tetramer (Figure 5). We conclude that the intermolecular disulphide bond is not required for the formation of a native tetramer. The small difference between the molecular mass of native β -glucuronidase, when calculated from our SDS/PAGE data (subunit molecular mass 83 kDa), and the value obtained by gel chromatography may be explained by the assumption that β -glucuronidase differs in its molecular shape from the protein standards used to calibrate the column. As gel-filtration chromatography measures molecular size rather than molecular mass, asymmetrical proteins may be eluted with an abnormally high molecular mass when compared with globular standard proteins.

For wild-type β -glucuronidase, two species with apparent molecular masses of 390 kDa and 190 kDa were obtained on gel filtration (Figure 5). The calculated molecular masses correspond to molecules that are composed of tetramers and dimers respectively. When tested for activity with the synthetic substrate 4-methylumbelliferyl β -D-glucuronide both species turned out to be enzymically active (results not shown). Samples of each protein peak were taken, stored overnight at 4 °C and run again on the same column. Only one peak corresponding to the one from which the protein originated appeared, indicating that neither protein species was in a reversible equilibrium (results not shown). The two species showed no differences in pH optimum, thermal stability, K_m value or specific activity.

For further characterization, samples from each protein peak were taken and analysed by SDS/PAGE. As shown in Figure 6, subunits of 83 kDa constitute the tetramer, whereas the dimer is composed mainly of proteolytically processed subunits of 79 kDa and, depending on the preparation, of an additional fragment of 64 kDa. These results demonstrate that proteolysis of β -glucuronidase affects the association of subunits. To determine whether the signal peptide was cleaved correctly and whether the 3–4 kDa loss from the processed subunits occurred from the N-terminus or the C-terminus, we carried out partial N-terminal sequencing. The sequence of the first six amino acids detected in both the 83 kDa and 79 kDa peptides was identical with that of the enzyme after cleavage of the signal peptide, as deduced from its cDNA. This result shows that the recombinant product is correctly processed at the N-terminus by the corresponding

Figure 7 Biosynthesis of wild-type and mutant β -glucuronidase in BHK cells

BHK cells transfected with wild-type and mutant cDNA constructs were pulse-labelled for 30 min with [35 S]methionine followed by a chase with unlabelled methionine for the time (h) indicated at the top of the lanes. Labelled proteins in the cell or the media were immunoprecipitated with goat anti- β -glucuronidase serum, and the products were subjected to SDS/PAGE. Wild-type (a and b) and mutant (c and d) enzymes were analysed under reducing (a and c) and non-reducing (b and d) conditions. NGS, normal goat serum control of BHK cells transfected with wild-type β -glucuronidase.

endoplasmic signal peptidase and locates the second proteolytic cleavage, the 3–4 kDa peptide loss, to the C-terminus of the protein. In dimer preparations that contained a 64 kDa fragment in addition to the 79 kDa peptide, a second N-terminal amino acid sequence was observed, showing that the 64 kDa component was derived from the 79 kDa component by cleavage between Val-159 and Gly-160. Cleavage at this site has been reported for β -glucuronidase purified from human placenta and chronic myelogenous leukaemic cells (Tanaka et al., 1992).

Intracellular transport and processing of wild-type and truncated human β -glucuronidase

A 3–4 kDa decrease in molecular mass as observed for our purified wild-type enzyme has been previously reported for intracellular β -glucuronidase of a number of mammalian species (Brown et al., 1981; Rosenfeld et al., 1982; Powell et al., 1988). For porcine kidney cells it has been shown that the proteolytic event occurred at the C-terminus (Erickson and Blobel, 1983). In order to follow the fate of recombinant β -glucuronidase that remains intracellular after synthesis, we labelled B702 and

B688 cells metabolically. Pulse-chase experiments are presented in Figure 7. Transfected BHK cells were labelled with [35 S]methionine for 30 min (pulse) and medium was collected and cells were harvested either directly or after a subsequent period of 0.5, 1, 3 or 8 h (chase). After 8 h of chase, half of the intracellular wild-type β -glucuronidase was processed to a slightly smaller form of 79 kDa (Figure 7a, lane 5). The secreted enzymes corresponded to the precursor form and consisted of disulphide-linked dimers (Figure 7b, lanes 6–10). The mutant, which is made in greater amounts, was processed as the wild-type enzyme and was equally well secreted (Figures 7c and 7d).

In order to determine whether the intracellular form of β -glucuronidase is a dimer, we lysed transfected BHK cells, removed nuclei and cell debris by centrifugation and loaded the supernatant on a TSK 3000 column for h.p.l.c. Enzyme activity of β -glucuronidase as well as the elution pattern on h.p.l.c. were not disturbed by a Nonidet P40 (0.5%, v/v)-containing lysis buffer (results not shown). Concentrated tissue culture supernatant of B702, BHK lysate mixed with purified β -glucuronidase from B702, B702 lysate and B688 lysate were separately run on a TSK G3000 column. As shown in Figure 8(a), β -glucuronidase

Figure 8 H.p.l.c. (TSK G3000) of intracellular human β -glucuronidase from transfected BHK cells

(a) Intracellular proteins of BHK cells expressing wild-type β -glucuronidase (clone B702) were subjected to h.p.l.c. Fractions were collected and tested for enzyme activity (.....). The elution profile of wild-type β -glucuronidase purified from B702 supernatant remains unchanged when mixed with BHK cell lysate before h.p.l.c. (—). The positions of tetramers and dimers are indicated. Enzyme activity of concentrated B702 supernatant is eluted mainly at the tetramer position (----). (b) After h.p.l.c. and SDS/PAGE, immunoblotting was used to analyse pooled fractions 20–23 (lane 1) and fractions 26–29 (lane 2) of purified β -glucuronidase and fractions 26–29 (lane 3) of intracellular β -glucuronidase.

from B702 supernatant was eluted as a single activity peak at the tetramer position. Purified B702 enzyme mixed with BHK cell lysate gave rise to two activity peaks corresponding to tetramer and dimer as observed in the absence of lysate. Intracellular wild-type and mutant β -glucuronidase from B702 (Figure 8a) and B688 lysate (results not shown), were eluted with a single activity peak at the dimer position. Cell lysate of untransfected BHK cells had no detectable β -glucuronidase activity under the conditions used. Western-blot analysis of the pooled fractions 26–29 of B702 lysate revealed that the majority of intracellular active β -glucuronidase corresponds to the processed form of purified β -glucuronidase (Figure 8b).

DISCUSSION

The β -glucuronidase of a variety of species has been extensively studied, and all previous reports describe the enzyme as a tetramer with its subunits held together by non-covalent forces (Lin et al., 1975; Tomino and Paigen, 1975; Owens and Stahl, 1976; Himeno et al., 1976; Brot et al., 1978; Diez and Cabezas, 1979; Jefferson et al., 1986). However, human β -glucuronidase secreted by transfected BHK cells turned out to be a tetramer

consisting of disulphide-linked dimers, and a truncated form of the enzyme demonstrates that Cys-644 is involved in disulphide-bond formation. These results suggest a dihedral symmetry for the oligomeric structure of the enzyme, i.e. a dimer of dimers, rather than cyclic symmetry, a notion that is supported by crystallographic data (Drendel et al., 1993).

Proteolytic processing is a normal post-translational event for intracellular β -glucuronidase and includes removal of a C-terminal propeptide (Brown et al., 1981; Erickson and Blobel, 1983; Islam et al., 1993). The secreted form of β -glucuronidase corresponds to the uncleaved precursor, indicating that the protein diverges from the lysosomal pathway before reaching a prelysosomal compartment, where processing is assumed to take place (Gabel and Foster, 1987). Although human β -glucuronidase was secreted by BHK cells exclusively in precursor form and purification was achieved by rapid and simple one-step immunoaffinity chromatography directly from tissue culture supernatant, some of the purified proteins were cleaved into smaller fragments. The respective amounts of each band varied among different purifications. When the 83 kDa band dominated, almost no 64 kDa band was detectable. The distinct proteolytic cleavage sites argue for exposed regions susceptible to proteases that originate most likely from the cells used for expression. With molecular masses of 79 kDa, 64 kDa and 18 kDa, the fragments generated from the 83 kDa precursor resemble those observed after purification of β -glucuronidase from human placenta (Brot et al., 1978; Tanaka et al., 1992). Isolation and partial N-terminal sequencing of both the 83 kDa precursor and 79 kDa mature form revealed that their N-termini are identical and correspond to the natural N-terminus of the enzyme after cleavage of the signal peptide. As in our mutant β -glucuronidase with a C-terminal truncation, the processed subunits do not migrate as dimers on SDS/PAGE, demonstrating that the processing event directly affects the pairwise covalent linkage of subunits. However, the functional significance of the disulphide bond is not understood, as the mutant enzyme is still able to form a tetramer, has the same activity as wild-type β -glucuronidase and its intracellular transport is not impaired.

Similar results with respect to tetramerization, transport and enzyme activity have been obtained by Sly and co-workers in a COS-cell expression system for a C-terminal-truncated version of human β -glucuronidase (Islam et al., 1993). In their mutant the last 18 amino acids after Thr-633 were deleted so that the C-terminus corresponded to the natural cleavage site of the propeptide found for the processed enzyme isolated from human placenta. Referring to sequence similarities between the murine propeptide and the reactive-site region of the serpin superfamily in murine β -glucuronidase, a potential cleavage site has been proposed to reside between Gly-637 and Ser-638 (Li et al., 1990). Therefore, without knowing the precise cleavage site, we eliminated in our mutant B688 only 14 amino acids from the C-terminus by introduction of a stop codon after the codon for His-637. When the relative mobility of wild-type and mutant β -glucuronidase is compared, it appears that the mutant migrates slightly faster, reflecting the loss of 14 amino acids. The fact that the processed 79 kDa fragment runs considerably faster than the mutant implies that more than 14 amino acids were removed from the C-terminus. Also cleavage after Thr-633 would not suffice to explain the increase in mobility of the processed form and we conclude that the proteolytic event in BHK cells removes a sequence including the glycosylation site at Asp-631.

All four potential N-linked glycosylation sites in human β -glucuronidase are assumed to be glycosylated (Shipley et al., 1993a). Indeed, evidence that proteolytic cleavage is accompanied by loss of a sugar moiety originates from the observation that on

Figure 9 Amino acid sequence, secondary structure and particular amino residues of the C-terminus of human β -glucuronidase

Amino acids 599–651 of the C-terminus of human β -glucuronidase (Oshima et al., 1987) are shown. Using the computer program Mac Vector (International Biotechnologies, Inc.), the secondary structure of β -glucuronidase was calculated according to the Chou–Asman method (CF) and the algorithm of Robson–Garnier (RG). Regions where secondary-structure predictions of both methods agree are depicted as black bars (CFRg). The arrow (↑) shows a site in human β -glucuronidase of proposed similarity to the reactive-site centre of serpins (Li et al., 1990) and indicates the beginning of the sequence deleted to generate a C-terminal-truncated mutant form of β -glucuronidase. The arrowhead (▲) indicates the site where proteolytic processing of a C-terminal propeptide was observed in human placental β -glucuronidase (Islam et al., 1993). Special amino acids are marked: Ala-619 to Val exchange (○) and Trp-627 to Cys (+) exchange results in mucopolysaccharidosis type VII (Tomatsu et al., 1990; Shipley et al., 1993b); cysteine-644 (—) is involved in disulphide-bond formation and Asn-631 (■) carries an oligosaccharide chain (Shipley et al., 1993a).

treatment with *N*-glycosidase F, the decrease in molecular mass for the unprocessed form is larger than for the mature form. The same effect of deglycosylation has been observed for rat β -glucuronidase expressed in COS cells (Powell et al., 1988). N-Terminal sequencing revealed a second proteolytic cleavage site between Val-159 and Gly-160 which the recombinant enzyme shares with β -glucuronidase isolated from human placenta (Tanaka et al., 1992). The deduced discrepancy between the C-terminus of recombinant versus natural β -glucuronidase may be explained by similar, yet different, proteolytic processing of the human enzyme in BHK cells.

A major structural implication of the C-terminal processing event in BHK cells is the tetramer–dimer conversion of the oligomeric structure of β -glucuronidase. The fact that enzymically active dimers of β -glucuronidase have not been observed previously may also argue for BHK-cell-specific C-terminal proteolytic processing of the enzyme. Our current knowledge about the C-terminus of β -glucuronidase is summarized in Figure 9. An exo- β -glucuronidase with a molecular mass of 130 kDa and a substrate specificity for the non-sulphated glycosaminoglycans, but not for *p*-nitrophenyl β -D-glucuronide, has been observed in rabbit liver (Nakamura, 1990). However, no data about the subunit size are provided and the authors surmise that the enzyme is not a different aggregation state of β -glucuronidase acting on *p*-nitrophenyl β -D-glucuronide but is a distinct protein.

Regardless of whether tetramer–dimer conversion is confined to certain as yet unidentified cell types or does not occur at all under physiological conditions, our findings provide valuable information about the oligomeric structure of β -glucuronidase. Some questions concerning the role of subunits in oligomeric protein function (Eisenstein and Schachman, 1989) are readily answered for β -glucuronidase: (i) the observation of enzymically active dimers supports the notion of the dihedral structure of the tetramer; (ii) the joint participation of amino acid residues from all four subunits is not required for enzyme activity; (iii) the forces that stabilize the tetramer are predominantly conferred by a C-terminal structure of the enzyme.

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