Active Site Residues of Human β-Glucuronidase

EVIDENCE FOR GLU540 AS THE NUCLEOPHILE AND GLU451 AS THE ACID-BASE RESIDUE*

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Human β-glucuronidase (hGUSB) is a member of family 2 glycosylhydrolases that cleaves β-d-glucuronic acid residues from the nonreducing termini of glycosaminoglycans. Amino acid sequence and structural homology of hGUSB and Escherichia coli β-galactosidase active sites led us to propose that residues Glu451, Glu540, and Tyr504 in hGUSB are involved in catalysis, Glu451 being the acid-base residue and Glu540 the nucleophile. To test this hypothesis, we introduced mutations in these residues and determined their effects on enzymes expressed in Cos cells and GUSB-deficient fibroblasts. The extremely low activity in cells expressing Glu451, Glu540, and Tyr504 hGUSBs supported their roles in catalysis. For kinetic analysis, wild type and mutant enzymes were produced in baculovirus and purified to homogeneity by affinity chromatography. The kcat/Km values (mM−1 s−1) of the E540A, E451A, and Y504A enzymes were 34,000-, 9100-, and 830-fold lower than that of wild type hGUSB, respectively. High concentrations of azide stimulated the activity of the E451A mutant enzyme, supporting the role of Glu451 as the acid-base catalyst. We conclude that, like their homologues in E. coli β-galactosidase, Glu540 is the nucleophilic residue, Glu451 the acid-base catalyst, and Tyr504 is also important for catalysis, although its role is unclear.

All three residues are located in the active site cavity previously determined by structural analysis of hGUSB.

Lyssosomal β-glucuronidase (EC 3.2.1.31) is an essential catalytic enzyme that is involved in the degradation of sulfated glycosaminoglycans. Deficiency of β-glucuronidase (GUSB) in humans produces a mucopolysaccharidosis type VII (Sly syndrome) (1, 2). In the absence of GUSB, chondroitin sulfate, dermatan sulfate, and heparan sulfate are only partially degraded and accumulate in the lysosomes of many tissues. The GUSB enzyme, synthesized as an 80-kDa glycoprotein monomer precursor (653 amino acids), is processed to a 78-kDa monomer by proteolytic cleavage, removing 18 amino acids from the C terminus (3, 4). Mature GUSB is normally a homotetramer, but there is evidence that the homodimer can also be enzymatically active (5).

Glycosidases function by using one of two general mechanisms leading either to retention or inversion of the anomeric configuration at the hydrolysis site (6, 7). In either case, two acidic residues, usually two glutamic acids, participate directly in catalysis. One amino acid acts as a catalytic nucleophile and the other as an acid-base catalyst or the proton donor. A number of mutations in hGUSB result in complete to partial loss of in vitro activity and have been associated with different disease phenotypes (8–12). However, none of these amino acids have been established as essential to the catalytic mechanism.

Based on the effects of salt, pH, and group-specific chemical reagents on the activity, Wang and Touster (19) proposed that a carboxylic acid and a carboxylate anion are the catalytic functional groups. Another approach to predict candidate catalytic residues is by sequence comparison with homologous enzymes whose active site residues have been identified. According to a recent classification based on amino acid sequence similarity, hGUSB was placed into family 2 together with Escherichia coli β-galactosidase (EGAL) (13, 14). X-ray crystal structure (15), inhibitor studies (16), and site-directed mutagenesis (17) studies of EGAL unequivocally established that the important catalytic residues include Glu537 as the nucleophile and Glu461 as the acid-base catalyst. Tyr503 was also found to be important for catalysis, but its role is not yet clear (18). From a sequence comparison of hGUSB with EGAL and a number of additional bacterial β-galactosidases, the candidate residues which correspond to the Glu537, Glu461, and Tyr503 in EGAL were identified as Glu540, Glu451, and Tyr504, respectively, in hGUSB (Fig. 1). Hydrophobic cluster assay, where homologous folds in glycosidases within the same family were compared, also predicted that Glu540 might be the nucleophile, whereas Glu451 might be the acid catalyst in hGUSB (20).

On the other hand, by comparing the x-ray crystal structure of hGUSB with those of lysozyme and EGAL, Jain et al. (21) proposed that the Asp207-Glu451 pair might form the nucleophile-acid-base catalyst pair in hGUSB, analogous to the Glu358–Asp372 pair in lysozyme.

In this report, we modified all the candidate residues proposed to be important for catalysis in hGUSB using site-directed mutagenesis. From the enzymatic activity and kinetic analyses, we concluded that Glu540-Glu451, not Asp207–Glu451, forms the nucleophile-acid catalyst pair in hGUSB. Furthermore, Tyr504, the residue analogous to Tyr503 in EGAL and also

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The abbreviations used are: GUSB, β-glucuronidase; hGUSB, human GUSB; EGAL, E. coli β-galactosidase; PAGE, polyacrylamide gel electrophoresis.

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located in the active site cavity of hGUSB (20), is also important for catalysis.

EXPERIMENTAL PROCEDURES

Materials—M13mp18, DEAE dextran, and nucleotides for DNA sequencing were from Amersham Pharmacia Biotech. Enzymes for molecular biology were from Omega and Promega, except for Sequenase®, which was from U. S. Biochemical Corp. Chloroquine and 4-methylumbelliferyl-β-glucuronide were from Sigma. LipofectAMINE was from Life Technologies, Inc. Tissue culture medium was from Life Technologies, Inc. Tran35S-label was from ICN, IgGusb was from the Enzyme Center (Malden, MA), and ENMANCE was from NEN Life Science Products. The biocinchoninic acid protein assay kit was from Pierce.

Construction of Mutant cDNAs—The mutations were generated with a single-strand mutagenesis system from Amersham Pharmacia Biotech in M13 vector and with a double-strand system using CLONTECH Corp. in pbLueScript (pBS KS) vector. In both vectors, a full-length hGUSB cDNA had been cloned into the EcoRI site. The mutant oligonucleotides (antisense) were as follows: E451A, GTG GCC AAC G CCT GCG TCC; E415Q, GTG GCC AAC CAG CGT GCG TTT; Y504F, GAA CAG CTG TTG ATT CAG TGT C; Y504H, GAA CAG CTG TTG ATT CAG TGT C; Y504A, GAA CAG CTG TTG ATT CAG TGT C; Y508A, GAA CAG CTG TTG ATT CAG TGT C; D207A, CAT ATT TCG CCT TCT TCA ACT ACG.

All mutant fragments except D207A, a 428-base pair fragment between SacII (1322) and SacI (1750) was excised from the respective mutant clone and swapped with the wild type fragment in human cDNA that had been previously cloned into the EcoRI site of expression vector pJC119RII (22). For the D207A mutant, a 262-base pair fragment generated by digestion with Apol (478) and BglII (740) was exchanged with the normal fragment between Apol-BglII in pJC119RII vector. All mutant fragments transferred into the wild type were verified to exclude undesired mutations by DNA sequencing using the dyeoxy chain termination method (23). The full-length mutant cDNAs constructed in this way were subcloned into the EcoRI site of expression vector pCAGGS (24) or Backpack-8, a baculovirus transfer vector (25).

Transfection, Metabolic Labeling, and Immunoprecipitation—COS-7 cells (26) were transfected with cDNAs in pCJ119RI using the DEAE-dextran method. Mouse GUSB-deficient 3521 cells were transfected with the cDNAs in pCAGGS using 6 μl of LipofectAMINE and 3 μg of plasmid DNA in a total volume of 200 μl in 25-mm Petri dishes. Media were collected, and cells were solubilized in 0.6 ml of 0.25% deoxycholic acid. Cell lysates and media followed by immunoprecipitation with anti-hGUSB antibody were assayed for catalysis.

Expression of wild type and mutant β-glucuronidase enzymes in COS and β-glucuronidase-deficient mouse fibroblasts 3521 cells

At 76 h after transfection, the media were collected, and the cells were lysed in 0.6 ml of 0.25% deoxycholic acid. Cell lysates and media were assayed for β-glucuronidase activity by using 4-methylumbelliferyl-β-D-glucuronide. ND, not determined.

PREPARATIONS—β-glucuronidase activity was determined by using 4-methylumbelliferyl-β-D-glucuronide. One unit is the amount of activity that releases 1 nmol of 4-methylumbelliferone/h (28). Protein concentrations were measured by bichinchoninic acid protein assay kit according to the manufacturer’s instructions using bovine serum albumin as standard.

Heat Inactivation Experiments—Wild type and mutant enzymes in 0.5× heat inactivation buffer (40 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, and 10 mg/ml bovine serum albumin) were incubated at 68 °C for 0 h and assayed for β-glucuronidase activity (29). Kinetic—The pH profiles for wild type and mutant enzymes were determined by adding 10 μl of the enzymes to 100 μl of 12.5 mM 4-methylumbelliferyl β-D-glucuronide in the buffers of the respective pH levels (0.1 mM sodium acetate, pH 3–5.5, 0.1 M Tris-HCl, pH 6.0–8.0) followed by incubation at 37 °C for 30 min. The kinetic parameters were determined by assay in β-glucuronidase activity at 37 °C in 0.2 mM acetate buffer at the respective pH optima with 0.5, 1, 2, and 4 mM 4-methylumbelliferyl β-D-glucuronide. The Km and Vmax were obtained from a double-reciprocal plot of initial substrate concentration versus rate of product formed.

RESULTS

Expression in COS and 3521 Cells—The mutants constructed by changing the wild type residues Glu451, Tyr504, and Glu540 were E451A, E451Q, Y504A, Y504H, Y504F, E540A, E540Q, and E540D. For comparison, we also made changes in the nucleophile and acid-base catalyst, producing E515A, and Y508H. All of these mutants were transiently expressed in COS7 cells with Cosmee Glu-Blue R-250. Protein concentrations were measured by bichinchoninic acid protein assay kit according to the manufacturer’s instructions using bovine serum albumin as standard.

Heat Inactivation Experiments—Wild type and mutant enzymes in 0.5× heat inactivation buffer (40 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, and 10 mg/ml bovine serum albumin) were incubated at 68 °C for 0 h and assayed for β-glucuronidase activity (29). Kinetic—The pH profiles for wild type and mutant enzymes were determined by adding 10 μl of the enzymes to 100 μl of 12.5 mM 4-methylumbelliferyl β-D-glucuronide in the buffers of the respective pH levels (0.1 mM sodium acetate, pH 3–5.5, 0.1 M Tris-HCl, pH 6.0–8.0) followed by incubation at 37 °C for 30 min. The kinetic parameters were determined by assay in β-glucuronidase activity at 37 °C in 0.2 mM acetate buffer at the respective pH optima with 0.5, 1, 2, and 4 mM 4-methylumbelliferyl β-D-glucuronide. The Km and Vmax were obtained from a double-reciprocal plot of initial substrate concentration versus rate of product formed.

### Table I

| cDNA | COS-7 cells | Wild type | 3521 cells | Wild type |
|------|-------------|-----------|------------|-----------|
|      | Total units/mg of β-glucuronidase expressed | % | Total units/mg of β-glucuronidase expressed | % |
| Wild type | 5926 | 100 | 666 | 100 |
| E540A | 0 | 0 | 0 | 0 |
| E515A | 4913 | 82.9 | ND | ND |
| E451A | 38 | 0.6 | 4 | 0.6 |
| D207A | 90 | 1.50 | 33 | 4.9 |
| Y504A | 10 | 0.56 | 4 | 0.56 |
| Y508A | 871 | 14.7 | ND | ND |
| E540D | 28 | 0.4 | 0 | 0 |
| E540Q | 7 | 0.1 | 2 | 0.3 |
| E541Q | 89 | 1.50 | 40 | 5.9 |
| Y504F | 6 | 0.10 | 15 | 2.3 |
| Y504H | 5 | 0.10 | 4 | 0.6 |

*Units/mg of expressed β-glucuronidase includes the total β-glucuronidase measured in cell extract and medium from which the endogenous COS cell enzyme (vector only transfections) in cell extract (124 units/mg) plus media (24 units/mg cell protein) was subtracted. The endogenous activity in W3521 cells and media was less than 1 unit/mg cell protein.
enzymes, the 3521 cell transfections with wild type and mutant cDNAs expressed in pCAGGS vector allow characterization of low activity mutant enzymes (24).

Table I shows that different mutants of Glu$^{540}$, the residue homologous to nucleophilic residue Glu$^{537}$ in EGAL, all had greatly reduced residual activity when expressed in COS cells and 3521 cells. The D207A mutant, which Jain et al. (21) had suggested might be the nucleophile, had 1.5% (COS cells) and 4.9% (3521 cells) of wild type activity, more than would be expected if it were the nucleophile residue.

The E451A mutant transfections produced only 0.6% of wild type activity in both cell types, whereas the E451Q mutant produced 1.5% (COS cells) and 5.9% (3521 cells) of wild type activity. These low activities are consistent with Glu$^{451}$ being the acid-base catalyst. The higher activity of E451Q could be explained by a low level of lysosomal deaminase activity converting the Q to E. These results contrast with the relatively high activity of the E515A mutant, which excludes it as an important residue in catalysis.

The three different mutants of Tyr$^{504}$, which is homologous to the required 503 in EGAL, had activities ranging from 0.1–0.5% in COS cells and 0.6–2.4% in 3521 cells. By contrast, Y508A had 14.7% of the wild type activity in COS cells. Taken together, these data suggest that Glu$^{540}$, Glu$^{451}$, and Tyr$^{504}$ in hGUSB have comparable roles in catalysis as their homologues in EGAL, i.e. that Glu$^{540}$ is the nucleophilic residue, Glu$^{451}$ is the acid-base catalyst, and Tyr$^{504}$ is also important for catalysis like Tyr$^{503}$ in EGAL, whose role in catalysis is not yet defined, although it is clearly located in or near the active site in EGAL (15) as is Tyr$^{504}$ in the active site of GUSB (21).

To compare synthesis, processing, and secretion of wild type and mutant enzymes, we carried out metabolic labeling of transfected COS cells. As shown in Fig. 2, when labeled for 1 h, the biosynthesis of all the mutant enzymes tested appeared comparable with that of the wild type. After a 24-h chase, all but one mutant (E540D) showed processing to the mature form (which is known to involve removal of the C-terminal 18 amino acid residues) and were secreted in amounts comparable to that of the wild type enzyme. E540D was exceptional. Even though some of the E540D enzyme was secreted, no processed enzyme was evident, and the intracellular enzyme appeared to be more rapidly degraded (Fig. 2, lanes 5 and 6). These observations could mean that some of the E540D mutant enzyme was retained in the endoplasmic reticulum and underwent endoplasmic reticulum-mediated degradation (31) or that it was rapidly degraded after delivery to lysosomes. The fact that the other mutants were processed normally to the mature form and secreted into the medium indicated that they were properly folded and were recognized by receptors in the secretory and lysosomal targeting pathways and by the processing enzyme(s) in endosomes and/or lysosomes.

**Purification and Kinetic Parameters**—To purify and characterize the mutant enzymes without contaminating wild type endogenous enzyme, the E540A, E451A, and Y504A mutants were transferred to the baculovirus genome by homologous recombination and produced in SF21 insect cells. The enzymes secreted into the growth medium were purified by affinity chromatography. As shown in Fig. 3, the purified mutant enzymes had the same migration patterns on SDS-PAGE as the wild type, except for Y504A, which had slightly faster migration. This faster mobility of Y504A could be due to a different level of glycosylation in SF21 cells. In fact, when the mutant enzyme was expressed in COS cells (Fig. 2, lanes 19–21), it showed the same mobility as the wild type enzyme. Size exclusion chromatography on TSK gel revealed that all three mutant enzymes were tetrameric (not shown).

The kinetic parameters obtained from the assays of the purified wild type and mutant enzymes are presented in Table II. The $K_m$ values were decreased 33,000-fold in E540A, 9,100-fold in E451, and 330-fold in Y504A mutant enzymes. However, the $K_m$ values were similar to the wild type hGUSB. These kinetic studies are consistent with our assignments of Glu$^{540}$ as the nucleophile, Glu$^{451}$ as the acid-base catalyst, and Tyr$^{504}$ as an important active site residue whose role is still not established.

**pH Optima and Heat Stability**—The enzyme activities of the wild type and mutant enzymes at different pH levels are shown in Fig. 4. Y504A had a broad pH activity profile between pH 3.0 and 8.0, similar to that of the wild type enzyme. The E540A mutant enzyme had an optimum at pH 5.0 instead of 4.5. The
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TABLE II

Kinetic values of E540A, E451A, and Y504A mutant enzymes

Measurements were made with 4-methylumbelliferyl-β-D-glucuronide at 37 °C. K_m values were calculated from double-reciprocal plots of reaction velocity versus substrate concentration. All the proteins including the wild type were produced in baculovirus and purified by affinity chromatography using a monoclonal antibody tresyl column.

| Constant | Wild type | E540A | E451A | Y504A |
|----------|-----------|-------|-------|-------|
| K_m (mM) | 2.76      | 1.20  | 1.33  | 1.67  |
| k_cat (s^-1) | 276  | 0.904 | 0.155 | 0.20  |
| k_cat/K_m (mM^-1 s^-1) | 100  | 0.003 | 0.011 | 0.12  |
| Fold decrease | 33,000 | 9,100 | 830  |       |

E451A enzyme showed a broader pH optimum (pH 4.0–5.0), and its activity drop with increasing pH was more gradual than that of wild type GUSB. It retained 35% of its activity at pH 8.

FIG. 4. pH profiles of the wild type (Wt) and mutant enzymes. The enzymes were produced in SF21 cells using a baculovirus system and purified as in Fig. 3. Enzymes were assayed in 1-h incubations with 4-methylumbelliferyl-β-D-glucuronide as substrate in buffer at the pH levels specified. The activities shown on the y axis are expressed as the maximum percentage of activity obtained for that enzyme.

FIG. 5. Heat inactivation of the wild type and mutant enzymes. The enzymes were produced in SF21 cells using a baculovirus system and purified as in Fig. 3. Enzymes were assayed in 1-h incubations without and with increasing sodium azide concentrations. Such inhibition was not noted by MacLeod et al. (32), and its basis is unclear. Like the wild type enzyme, the E451A mutant enzyme also showed inhibition by azide and lost 70% of its original activity in 50 mM azide. However, concentrations of azide between 50 mM and 0.5 M stimulated activity of the E451A enzyme. Activity was 4-fold greater at 500 mM azide than that seen at 50 mM azide. Further increase in azide concentration to 1 M inhibited the E451A enzyme like the wild type enzyme. Azide had no effect on the extremely low activity of the E540A mutant enzyme.

FIG. 6. Azide enhancement of the activity of the E451A mutant enzyme. Purified wild type (Wt) and mutant (E451A and E540A) enzymes were measured in 1-h incubations without and with increasing concentrations of sodium azide.

DISCUSSION

Recent classification of glycosyl hydrolases based on comparison of amino acid sequences in the active sites placed hGUSB into family 2 together with EGAL (6, 7). The active site of the latter has been studied in great detail, and two glutamate/glutamic acid residues (Glu^537 and Glu^451) were identified to be involved in catalysis (15–18). Recently, the three-dimensional structure of EGAL confirmed that Glu^537 and Glu^451 are in the active site cleft and positioned at a distance that would be consistent with them forming the nucleophile and acid-base catalyst pair and their participating in the retaining type catalysis. In addition, early mutational studies had identified Tyr^503 in EGAL as an important catalytic residue. Although structural studies show that Tyr^503 forms part of the active site cavity, its role in catalysis is still unknown (16). Amino acid sequence comparison of hGUSB with mouse, rat, or E. coli GUSB and EGAL revealed the three residues in hGUSB that correspond to Glu^537, Glu^451, and Tyr^503 in EGAL to be Glu^540, Glu^461, and Tyr^504, respectively (Fig. 1).

The data presented here support the predictions based on homology to active site residues in EGAL and those based on hydrophobic cluster analysis (20) implicating Glu^540 and Glu^451 as the nucleophile/acid-base pair involved in catalysis of hGUSB. Furthermore, based on alignment of a variety of retaining-type glycosyl hydrolases (32) and on hydrophobic cluster assay of regions surrounding the catalytic amino acids...
identified for a few retaining -glycosyl hydrolases, similar motifs were present in over 150 glycosyl hydrolases. In all for which the nucleophile residue has been identified, the putative proton donor (acid-base catalyst) is located upstream of the nucleophile and is preceded immediately by an invariant Asn residue and also preceded by a conserved Trp residue five residues upstream. In GUSB, Glu\(^{451}\) is upstream of Glu\(^{440}\) and is located in the sequence WSVANEP.

Another piece of evidence consistent with Glu\(^{451}\) being the acid-base residue is the increase in activity of the E451A mutant enzyme in the presence of azide. MacLeod et al. (32) suggested from the increase in \(k_{\text{cat}}\) with some substrates seen with E127A mutant exoglucanase/xylanase in the presence of 60 mM to 2 mM azide that azide can occupy a vacant anionic site created by removal of the acid-base catalyt (E127A in their case and E451A in GUSB) and react rapidly with the glucosyl-enzyme intermediate, increasing the steady state rate and forming the glycosyl azide product. We interpret the stimulation of E451A hGUSB by 50-500 mM azide to support its role as the acid-base catalyst, although inhibition of the wild type enzyme by azide, the mechanism of which is not yet clear, complicates the interpretation of this experiment. Furthermore, it has been noted in the retaining type glycosyl hydrolases for which crystal structures are available (20) that both active site residues are located in the C-terminal TIM barrel. The x-ray crystal structure of hGUSB placed residues Glu\(^{451}\) and Glu\(^{440}\) in the C-terminal TIM barrel formed by residues 343-642 and located both residues in the active site cleft (21).

Using another approach to characterize the mechanism of hydrolysis of GUSB and to identify its active site residues, Wong et al. (35) recently determined that hGUSB is a retaining acid hydrolase using NMR analysis of the product of hydrolysis. In addition, by analysis of the labeled peptides after hydrolysis of GUSB and to identify its active site residues, 343-642 and located both residues in the active site cleft (21).

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