Identification of Glu-540 as the Catalytic Nucleophile of Human β-Glucuronidase Using Electrospray Mass Spectrometry

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Human β-glucuronidase (HGB) is a lysosomal exoglycosidase that cleaves β-D-glucuronic acid residues from the nonreducing termini of glycosaminoglycans. The enzyme is shown to catalyze glycoside bond hydrolysis with net retention of anomeric configuration, presumably via a mechanism involving a covalent glucuronyl enzyme intermediate. Incubation of human β-glucuronidase with 2-deoxy-2-fluoro-β-D-glucuronyl fluoride resulted in time-dependent inactivation of the enzyme through the accumulation of a covalent 2-deoxy-2-fluoro-α-D-glucuronyl-enzyme, as observed by electrospray mass spectrometry. Regeneration of the free enzyme by hydrolysis or transglycosylation and removal of excess inactivator demonstrated that the covalent intermediate was kinetically competent. Peptic digestion of the 2-deoxy-2-fluoro-α-D-glucuronyl-enzyme intermediate and subsequent analysis by liquid chromatography coupled with electrospray ionization triple quadrupole mass spectrometry indicated the presence of a 2-deoxy-2-fluoro-α-D-glucuronoyl peptide. Sequence determination of the labeled peptide by tandem mass spectrometry in the daughter ion scan mode permitted the identification of Glu-540 as the catalytic nucleophile within the sequence SEYGAET.

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‡ The abbreviations used are: HGB, human β-glucuronidase; 2-FGlcnUA, 2-deoxy-2-fluoro-β-D-glucuronic acid fluoride; HPLC, high pressure liquid chromatography; TEMPO, 2,2,6,6-tetramethyl-1-piperidinylxoy; pNPGlcUA, p-nitrophenyl β-D-glucuronide; MS, mass spectrometry; LC/MS, liquid chromatography/mass spectrometry.

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group, accelerates the first step, permitting the trapping of a 2-deoxy-2-fluoro-O-β-glycosyl-enzyme intermediate. Proteolytic digestion of the labeled enzyme yields a mixture of peptides, one of which has the fluoro-sugar attached. Isolation of this peptide, followed by tandem mass spectrometric analysis, permits identification of the nucleophile. This study presents evidence that HBG is a retaining glycosidase and describes the synthesis of 2-deoxy-2-fluoro-β-D-glucuronoyl fluoride (2-FGlcUAF) and its use in the identification of the enzyme’s catalytic nucleophile.

**EXPERIMENTAL PROCEDURES**

**General Methods**—All buffer chemicals and other reagents were obtained from Sigma/Aldrich unless otherwise noted. Selectfluor™ (1-chloromethyl-4-fluoro-1,4-diazobicyclo[2.2.2]octane bis(tetrafluoroborate)) was generously donated by Air Products & Chemicals Inc. Pepsin (from porcine gastric mucosa) was obtained from Boehringer Mannheim. Human β-glucuronidase was purified as described previously (17, 18).

**Synthesis of 2-Deoxy-2-fluoro-β-D-glucopyranosyl Uronic Acid Fluoride (6)**—Reactions were monitored by thin-layer chromatography using Merck Kieselgel 60 F254 aluminum-backed sheets. Compounds were detected by charring with either 10% ammonium molybdate in 2 M H2SO4 or 10% H2O2/methanol and heating. Flash chromatography under a positive pressure was performed with Merck Kieselgel 60 (230–400 mesh) using the specified eluents. 1H NMR spectra were recorded on a Bruker AC-200 spectrometer at 200 MHz (chemical shifts quoted relative to CDCl3 or 2,2-dimethyl-2-silapentane-5-sulfonic acid when taken in D2O). 19F NMR spectra were recorded on a Bruker AC-200 spectrometer at 188 MHz (chemical shifts quoted relative to CFCI3).

**Synthesis of 1,3,4,6-Tetra-O-acetyl-2-deoxy-2-fluoro-D-mannopyranose (31%).** The 1H NMR spectra were identical to those previously reported (19).

**Synthesis of 2-Deoxy-2-fluoro-β-D-glucopyranosyl Fluoride (4)**—The fluoride (4) was prepared by dissolving 3 g of tetraacetate (2) (8.6 mmol) in 45% HBr/glacial acetic acid (10 ml) and stirring at room temperature for 15 min. The mixture was then diluted with water (200 ml) and then treated with CH2Cl2 (2 × 150 ml). The organic phase was washed with water and saturated sodium bicarbonate, dried over magnesium sulfate, and concentrated in vacuo. The resulting syrup was dissolved in 30 ml of HPLC-grade acetonitrile. To the solution was added silver fluoride (2.17 g, 17.1 mmol), and the suspension was allowed to stir overnight in the dark. The silver salts were then filtered off through a silica gel plug using ethyl acetate as the eluent, and the solvents were removed in vacuo. The syrup was dissolved in anhydrous methanol (30 ml), and then sodium methoxide (46 mg, 0.86 mmol) was added to the solution. After stirring at room temperature for 30 min, the reaction mixture was neutralized with Amberlite IR-120 (H+ form) and concentrated in vacuo. Silica gel chromatography (27:2:1 ethyl acetate/methanol/water) yielded 1.34 g (85%) of compound 4. The 1H NMR spectrum was identical to that previously reported (19).

**Synthesis of Phenacyl (2-Deoxy-2-fluoro-β-D-glucopyranosyl Uronate (7)**—To a stirred solution of the 2-fluoroglucosyl fluoride (4) (19.5 mg, 0.106 mmol) in water (0.5 ml) was added sodium bromide (3.3 mg, 0.032 mmol) and TEMPO (0.5 mg, 0.0032 mmol). After cooling to 0 °C, 200 μl of water and extracted with CH2Cl2 (2 × 150 ml). The organic phase was dried over magnesium sulfate and concentrated in vacuo. Silica gel chromatography (27:2:1 ethyl acetate/methanol/water) yielded 1.34 g (85%) of compound 4. The 1H NMR spectrum was identical to that previously reported (19).

**Synthesis of Phenacyl (2-Deoxy-2-fluoro-β-D-glucopyranosyl Uronate Fluoride (5)**—To a solution of triethylamine (16 μl, 0.115 mmol) in 45% HBr/glacial acetic acid (10 ml) was added sodium bromide (3.3 mg, 0.032 mmol) and TEMPO (0.5 mg, 0.0032 mmol). After cooling to 0 °C, 200 μl of water and extracted with CH2Cl2 (2 × 150 ml). The organic phase was dried over magnesium sulfate and concentrated in vacuo. Silica gel chromatography (27:2:1 ethyl acetate/methanol/water) yielded 1.34 g (85%) of compound 4. The 1H NMR spectrum was identical to that previously reported (19).

**Determination of the Stereocchemical Course of Hydrolysis**—The buffer used in this experiment was 100 mM acetate solution, pH 4.8, in D2O (buffer A), prepared by adjusting the pH of a 100 mM solution of sodium acetate in D2O with a 100 mM solution of acetic acid in D2O. The 1H NMR spectrum of a sample of 1.1 mg (3.3 μmol) of p-nitrophenyl O-β-glucuronide (pNPGLcUA) in 400 μl of buffer A was recorded on a Bruker AC-200 spectrometer, and then HBG (200 μl, 0.95 mg/ml), previously exchanged with buffer A using 30-kDa nominal molecular mass cutoff centrifugal concentrators (Amicon Centricron-30), was added. 1H NMR spectra were recorded at different time intervals.

**Enzyme Kinetics**—Kinetic studies were performed at 37 °C. All studies on human β-glucuronidase were performed in 100 mM sodium acetate buffer, pH 4.8 (buffer B), a continuous spectrophotometric assay based on the hydrolysis of the pNP-glucuronides. NMR experiments were used to monitor enzyme activity by measurement of the rate of p-nitrophenolate release upon hydrolysis (λ = 360 nm, ε = 2.25 × 103 M−1 cm−1 in buffer B) using a Unicam 8700 UV-visible spectrophotometer equipped with a circulating water bath. Michaelis-Menten parameters for the substrate, previously unidentified with this enzyme, were Km = 1.56 mM and kcat = 250 min−1 at 37 °C in buffer B.
Inactivation Studies—The inactivation of human β-glucuronidase by 2-FGlcUAF was monitored by incubation of the enzyme (~0.2 mg/ml) under the above conditions in the presence of various concentrations of 2-FGlcUAF at 37 °C in a total volume of 100 μl. Residual enzyme activity was determined at the appropriate time intervals by addition of a 10-μl aliquot of the inactivation mixture to a solution of pNPGlcUA (1 mM, 700 μl) in buffer A and measurement of p-nitrophenolate release over a period of 1 min. Pseudo first-order inactivation rate constants at each inactivator concentration \( k_{\text{obs}} \) were determined by fitting each curve to a first-order equation using the program GraFit (27). The value of \( k_{\text{obs}} \) was monitored by removing aliquots (1 μl) at several time intervals and assaying for activity as described above. Approximately 50–75% of the activity was recovered relative to a control of native enzyme treated in an identical manner.

Labeling and Proteolysis—β-Glucuronidase (30 μl, 3.7 mg/ml) was incubated with 2-FGlcUAF (4 μl, 50 mM) at 37 °C for 10 min in buffer B; complete inactivation (>99%) was confirmed by enzyme assay as described above. This mixture was immediately subjected to peptic digestion: β-glucuronidase (10 μl of native or 34 μl of 2-deoxy-2-fluoro-α-D-glucuronol-enzyme, 3.7 mg/ml) was mixed with pepsin (10 or 34 μl, respectively; 0.4 mg/ml in 300 mM sodium phosphate, pH 2.0) and 300 mM sodium phosphate buffer, pH 2.0 (10 or 34 μl, respectively). The mixture was incubated at 37 °C for 1 h, frozen, and analyzed immediately by liquid chromatography/mass spectrometry (LC/MS) upon thawing.

Electrospray Mass Spectrometry—Mass spectra were recorded on a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with an ionspray ion source. Peptides were separated by reverse-phase HPLC on an Ultrafast Microprotein analyzer (Michrom BioResources Inc., Pleasanton, CA) directly interfaced with the mass spectrometer. In each of the MS experiments, the proteolytic digest was loaded onto a C18 column (Reliasil, 1 × 150 mm) equilibrated with solvent A (solvent A: 0.05% trifluoroacetic acid and 2% acetonitrile in water). Elution of the peptides was accomplished using a gradient (0–60%) of solvent B over 60 min, followed by 100% solvent B over 2 min (solvent B: 0.045% trifluoroacetic acid and 80% acetonitrile in water). Solvents were pumped at a constant flow rate of 50 μl/min. Spectra were obtained in the single quadrupole scan mode (LC/MS) or the tandem MS product ion scan mode.

In the single quadrupole mode (LC/MS), the quadrupole mass analyzer was scanned over a m/z range of 400–1800 Da with a step size of 0.5 Da and a dwell time of 1.5 ms/step. The ion source voltage was set at 5.5 kV, and the orifice energy was 45 V. In the tandem MS daughter ion scan mode, the spectrum was obtained by selectively introducing the parent ion (m/z = 935) from the first quadrupole (Q1) into the collision cell (Q2) and observing the product ions in the third quadrupole (Q3). Thus, Q1 was locked on m/z = 935; the Q3 scan range was 50–1120 Da; the step size was 0.5 Da; the dwell time was 1 ms; the ion source voltage was 5 kV; the orifice energy was 45 V; Q0 = −10 V; and IQ2 = −48 V.

**FIG. 2.** Proton NMR spectra showing the stereochemical course of hydrolysis of pNPGlcUA by HBG. Spectra are for the anomeric proton region of the substrate at different time intervals relative to addition of HBG: 0 min (A), 1.5 min (B), 4 min (C), and 20 min (D).
RESULTS AND DISCUSSION

Stereochemistry of HBG Hydrolysis—The use of proton NMR in the determination of the stereochemical course of enzyme-catalyzed glycoside hydrolyses has been demonstrated previously (20, 21). Chemical shifts and coupling constants of the anomeric protons of α- and β-glycosides and the product hemiacetals are distinct and readily observed. When sufficient enzyme is used to complete the hydrolysis quickly (typically, 2 min), the initially formed anomer is detected before mutarotation has occurred to any significant extent. Fig. 2 illustrates the experiment performed using HBG. Fig. 2A shows the anomeric proton region of pNPGlcUA in buffer A. The multiplet centered at δ 5.18–5.28 ppm arises from the axial anomeric proton of the β-glycoside substrate, and the large resonance at δ 4.7 ppm is from HOD. Panels B–D were recorded at time intervals after addition of the enzyme. As shown in Fig. 2B, the enzymatic hydrolysis was essentially complete after 1.5 min since the resonance at δ 5.18–5.28 ppm had almost disappeared. Simultaneously, a new resonance appeared at δ 4.58 ppm (J = 7.9 Hz). The chemical shift and coupling constant identify this as the axial anomeric proton of β-D-glucuronic acid. After 4 min, a new doublet appeared at δ 5.18 ppm (J = 3.6 Hz) (Fig. 2C). This corresponds to the equatorial anomeric proton of α-D-glucuronic acid resulting from mutarotation of the initial β-D-glucuronic acid product. After 20 min, the product D-glucuronic acid converted via mutarotation to the equilibrium ratio of anomers (34:66 α/β) (Fig. 2D). The data unequivocally demonstrate that hydrolysis catalyzed by HBG proceeds with net retention of anomeric configuration, presumably via a double displacement mechanism.

Synthesis of Inactivator—The synthesis of 2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride is outlined in Fig. 3. The key steps involved the use of Selectfluor™ to introduce fluorine at C-2 and the use of TEMPO in the selective oxidation of the C-6 alcohol to the carboxylic acid. Selectfluor™ is an electrophilic fluorinating agent that is both stable and easy to use (22). It reacts with olefins in the presence of a weak nucleophile such as water or acetic acid to yield the addition product, and it has previously been shown to react with glycals to yield a variety of 2-deoxy-2-fluoroglycosides (23). In particular, tri-O-acetyl-D-glucal was shown to react with Selectfluor™ in N,N-dimethylformamide/water to give predominantly the manno-epimer. We found that by changing the solvent/nucleophile system to acetic anhydride/acetic acid, the glucopyranosyl fluoride product was converted to 2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride.

![Figure 3](http://www.jbc.org)  
**Fig. 3.** Reaction scheme used in the synthesis of 2-deoxy-2-fluoro-β-D-glucopyranosyluronic acid fluoride (7). Arrow a, Selectfluor™, acetic anhydride, acetic acid; arrow b, 45% HBr/acetic acid; arrow c, AgF, acetonitrile; arrow d, NaOMe/MeOH; arrow e, TEMPO, NaOCl, NaBr; arrow f, phenacyl bromide, triethylamine, N,N-dimethylformamide; arrow g, 10% palladium on charcoal, H₂.

![Figure 4](http://www.jbc.org)  
**Fig. 4.** Inactivation of human β-glucuronidase by 2-FGlcUAF. A, semi-logarithmic plot of residual activity versus time at the indicated inactivator concentrations: 0.05 mM (●), 0.1 mM (○), 0.5 mM (□), 1 mM (■), 3 mM (▲), and 5 mM (▲). B, replot of the first-order rate constants from A. C, inactivation with 150 μM 2-FGlcUAF in the absence (○) and presence (●) of 0.68 μM d-saccharic acid 1,4-lactone.
by a series of steps involving bromination, fluoride displacement, and deprotection.

Glucopyranosyluronic acid was obtained via TEMPO-mediated oxidation of the C-6 alcohol of 2-deoxy-2-fluoro-\(\alpha\)-D-gluco-pyranosyl fluoride in water buffered at pH 10 with hypobromite (formed by the reaction of hypochlorite and bromide) as the regenerating oxidant (24–26). This reaction is both mild and selective for the primary C-6 alcohol. To facilitate purification of the product away from the inorganic salts present, the uronic acid was first converted to its phenacyl ester and then purified chromatographically. The choice of the phenacyl ester was based upon the need for a protecting group that can be easily removed under mild conditions, i.e. non-acidic and non-basic conditions. This particular ester is easily cleaved via catalytic hydrogenolysis.

**Inactivation Kinetics**—Incubation of HBG with 2-FGlcUAF resulted in inactivation of the enzyme in a time-dependent manner according to pseudo first-order kinetics (Fig. 4A). However, no saturation was observed, even at the highest inactivator concentrations studied (Fig. 4B); yet higher concentrations could not be investigated due to the rapidity of inactivation, which precluded accurate sampling. Reliable values for the inactivation rate constant \(k_i\) or the reversible dissociation constant \(K_i\) therefore could not be determined. However, a reliable second-order rate constant of \(k_i/K_i = 286 \text{ min}^{-1} \text{ M}^{-1}\) was calculated for the slope of the plot of \(k_{\text{obs}}\) versus [2-FGlcUAF].

Incubation of the enzyme with 2-FGlcUAF (150 \(\mu\)M) in the presence of the competitive inhibitor \(\beta\)-saaccharic acid 1,4-lactone (1) (0.68 \(\mu\)M) resulted in a two-phase reaction (Fig. 4C). The initial inactivation rate constant was calculated to be 0.054 \(\text{min}^{-1}\), nearly identical to the apparent inactivation rate constant of 0.057 \(\text{min}^{-1}\) obtained in the absence of the lactone. The final inactivation rate constant was calculated to be 0.019 \(\text{min}^{-1}\). This observed protection from inactivation in the pres-
ence of the lactone is consistent with 2-FGlcUAF being active site-directed. These results suggest that inactivation is a consequence of accumulation of a stable covalent 2-deoxy-2-fluoro-\(\alpha\)-D-glucuronyl-enzyme intermediate, a conclusion that is supported by the mass spectral analysis of the inactivated enzyme.

**Catalytic Competence**—Further evidence supporting the existence of a covalent 2-deoxy-2-fluoro-\(\alpha\)-D-glucuronyl-enzyme arises from demonstration of the catalytic competence of the trapped intermediate. Following removal of excess inactivator from the labeled enzyme, the sample was incubated at 37 °C in the presence of buffer B alone, with 50 mM chitobiose, or with 50 mM \(\text{N-acytelyglucosamine}\), and the recovery of activity associated with the regeneration of the free enzyme was monitored. Reactivation kinetics of the 2-deoxy-2-fluoro-\(\alpha\)-D-glucuronyl-enzyme in buffer alone followed a first-order process with an apparent rate constant of \(k_{\text{app}} \approx 0.0040 \text{ h}^{-1}\) (Fig. 5). Rate constants for reactivation by transglycosylation (\(k_{\text{trans}}\)) were found to be \(0.0033 \text{ h}^{-1}\) (\(k_{\beta} = 303 \text{ h}^{-1}\) with chitobiose). The higher enzyme reactivation rate observed in the presence of chitobiose (2-fold higher than the spontaneous reactivation rate) suggests that reactivation is accelerated by transglycosylation to an acceptor sugar. That chitobiose functions as a transglycosylation acceptor is not surprising since the natural substrates of HBG are the glycosaminoglycans, oligosaccharides composed of alternating glucuronic acid and \(\text{N-acetylglycosamine}\) residues, and chitobiose is a GlcNAc(\(\beta\)-1→4)GlcNAc disaccharide.

**Identification of the Labeled Active-site Peptide by Electrospray MS**—Peptic hydrolysis of native HBG or the 2-deoxy-2-fluoro-\(\alpha\)-D-glucuronyl-enzyme resulted in a mixture of peptides, which were separated by reverse-phase HPLC using the electrospray MS as detector. When scanned in the normal LC/MS mode, the total ion chromatograms showed a large number of peaks, each corresponding to one or more peptides in the digest mixture (Fig. 6, A and C). The masses under each peak in the labeled sample were compared with the masses of the corresponding peptides in the native sample, searching for a peptide present only in the labeled sample that was 178 Da greater than a peptide present in the unlabeled sample. Only one pair of peaks satisfied this requirement of difference in mass by that of the attached label, these being a peak at m/z 756 in the native digest that was not observed in the labeled digest (Fig. 6B) and a peak at m/z 934 in the labeled digest that was not observed in that of the native enzyme (Fig. 6D). The mass difference between these two peaks is 178 Da, which corresponds exactly to the mass of the 2-deoxy-2-fluoro-\(\alpha\)-D-glucuronyl label. The labeled parent ion (m/z 934) and the unlabeled intact peptide (m/z 756) thus appear as singly charged species.

Candidate peptides with a mass of 756 ± 2 Da were then identified by inspection of the amino acid sequence of the enzyme and searching for all possible peptides with this mass. Twenty such peptides were identified, but of these, all but five were eliminated because their sequences did not contain either an aspartate or a glutamate residue. Precedent with all retaining glycosidases to date would predict that the nucleophile should be one of these two amino acids. The candidate peptides are 257KLEVRL262 60EEQYW64 310DFTLP323 485NSNYAAD490, and 538SEYGAET445. The peptide was then unambiguously identified by peptide sequencing using tandem MS.

**Peptide Sequencing**—Information on the sequence was obtained by additional fragmentation of the peptide of interest (m/z 934) in the daughter ion scan mode (Fig. 7). The parent ion of interest (m/z 934) was selected in the first quadrupole and subjected to collision-induced fragmentation, and then the masses of the daughter ions were detected in the third quadrupole. Peaks resulting from Y′ ions correspond to fragments ET (m/z 249), AET (m/z 320), GAET (m/z 378), YGAET (m/z 540), and EYGAET (m/z 669). Peaks arising from B ions bearing the label include SE (m/z 395), SEY (m/z 558), SEYG (m/z 616), SEYGA (m/z 687), and SEYGAET (m/z 816). Because the B ions bearing the label include SE (m/z 395), we can infer that the label is linked to either Ser-539 or Glu-540. This information, in conjunction with the mass of the labeled peptide and the primary sequence of the enzyme, permits identification of the peptide containing the active-site nucleophile as 538SEYGAET445. Comparison of this sequence with those of the other Family 2 glycosidase sequences shows that the dipeptide Y′ is conserved only within a particular glycosidase category, i.e. galactosidase versus glucuronidase versus mannosidase. From this, we can assign Glu-540 as the catalytic nucleophile.

Doubts have been expressed previously concerning the identity of the catalytic nucleophile of HBG. In particular, on the basis of the three-dimensional crystal structure, it had been suggested that Asp-207 might well serve in this role (8). Furthermore, the fact that \(E.\ coli\ \beta\text{-glucuronidase}\) is a metalloenzyme, requiring a Mg\(^{2+}\) bound at the active site for catalytic activity, whereas HBG has no such requirement, causes concern about parallels drawn between the two enzymes’ active sites. These are further heightened by the fact that HBG cleaves a substrate containing a carboxylic acid, thus a different active-site composition may be required to accommodate this additional charge. However, the assignment of Glu-540 as the catalytic nucleophile of HBG removes any ambiguity concerning the identity of this residue and is completely consistent with expectations on the basis of sequence similarity (6).

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