Family 39 α-L-Iduronidases and β-D-Xylosidases React through Similar Glycosyl−Enzyme Intermediates: Identification of the Human Iduronidase Nucleophile†

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ABSTRACT: The inclusion of both β-D-xylosidases and α-L-iduronidases within the same sequence-related family (family 39), despite the considerable difference in substrate structures and poor sequence conservation around the putative nucleophile, raises concerns about whether a common mechanism is followed by the two enzymes. A novel anchimeric assistance mechanism for iduronidases involving a lactone intermediate is one possibility. NMR analysis of the methanolysis reaction catalyzed by human α-L-iduronidase reveals that, as with the β-D-xylosidases, α-L-iduronidase is a retaining glycosidase. Using two different mechanism-based inactivators, 5-fluoro-α-L-iduronyl fluoride and 2-deoxy-2-fluoro-α-L-iduronyl fluoride, the active site nucleophile in the human α-L-iduronidase was identified as Glu299 within the $_{295}$IYNDEAD$_{301}$ sequence. The equivalent, though loosely predicted, glutamic acid was identified as the nucleophile in the family 39 β-D-xylosidase from *Bacillus* sp. [Vocadlo, D., *et al.* (1998) *Biochem. J. 335,* 449−455]; thus, a common mechanism involving a covalent glycosyl−enzyme intermediate that adopts the rather uncommon $^{2,5}B$ conformation is predicted.

Mucopolysaccharidosis type I (MPS I)[1] is caused by the lack of the lysosomal enzyme α-L-iduronidase (IDUA). This 85 kDa enzyme is responsible for cleaving α-L-iduronic acid (IdoA) from the nonreducing ends of the glycosaminoglycan polymers heparin, heparan sulfate, and dermatan sulfate. Possible clinical outcomes include Hurler syndrome, for which symptoms include mental retardation, organomegaly, corneal opacity, and skeletal deformities (dwarfism). Those afflicted usually do not survive beyond 10 years of age. Another possible clinical outcome is Scheie syndrome, which is less severe; patients have normal mental capacity and life expectancy but still display the symptoms of skeletal deformities and corneal opacity. Until very recently, the only available treatment for the disease was allogenic bone marrow transplantation, but this treatment is limited by the availability of donors. Considerable research has been done into the use of enzyme replacement therapy, including the use of a knockout mouse model (*1*) and a naturally occurring canine model (*2*), culminating in the very recent approval of this approach by the U.S. Federal Drug Administration.

However, despite the push toward the use of iduronidase as a therapeutic agent, very little is known about the activity of iduronidase on a mechanistic level. One of the first clues about the mechanism of a glycosidase is derived from its assignment to a glycosidase family. On the basis of amino acid sequence homology, Henrissat has divided all known glycosidases into a set of families (*3*, *4*), and enzymes grouped together usually share a common mechanism. In 1990, the iduronidase gene was mapped to chromosome 4p16.3 (*5*, *6*), and in 1991, the cDNA was isolated and cloned (*7*). On the basis of its sequence, iduronidase was assigned to glycosyl hydrolase family 39, which is a small family, currently comprising only α-L-iduronidases and a small number of β-D-xylosidases. Interestingly, a high degree of sequence similarity is found among the iduronidases and also among the xylosidases, with only a lower level of similarity between the two groups of enzymes, making this family assignment somewhat suspect.

The stereochemical course of the reaction catalyzed by glycosidases is a primary criterion of the mechanism that is followed, and is generally expected to be the same for all glycosidases within a family. The first determination of such a stereochemical outcome for a family 39 enzyme was carried out in 1996 by Armand *et al.* (*8*) on a β-xylosidase isolated

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[1] Abbreviations: 5IFIdoAF, 5-fluoro-α-L-idopyranosyluronic acid fluoride; 2FIdoAF, 2-fluoro-α-L-idopyranosyluronic acid fluoride; IdoA, α-L-iduronic acid; IDUA, human lysosomal α-L-iduronidase; MPS I, mucopolysaccharidosis type I; MUI, 4-methylumbelliferyl α-L-iduronide; pNPIdoA, 4-nitrophenyl α-L-idopyranosiduronate.

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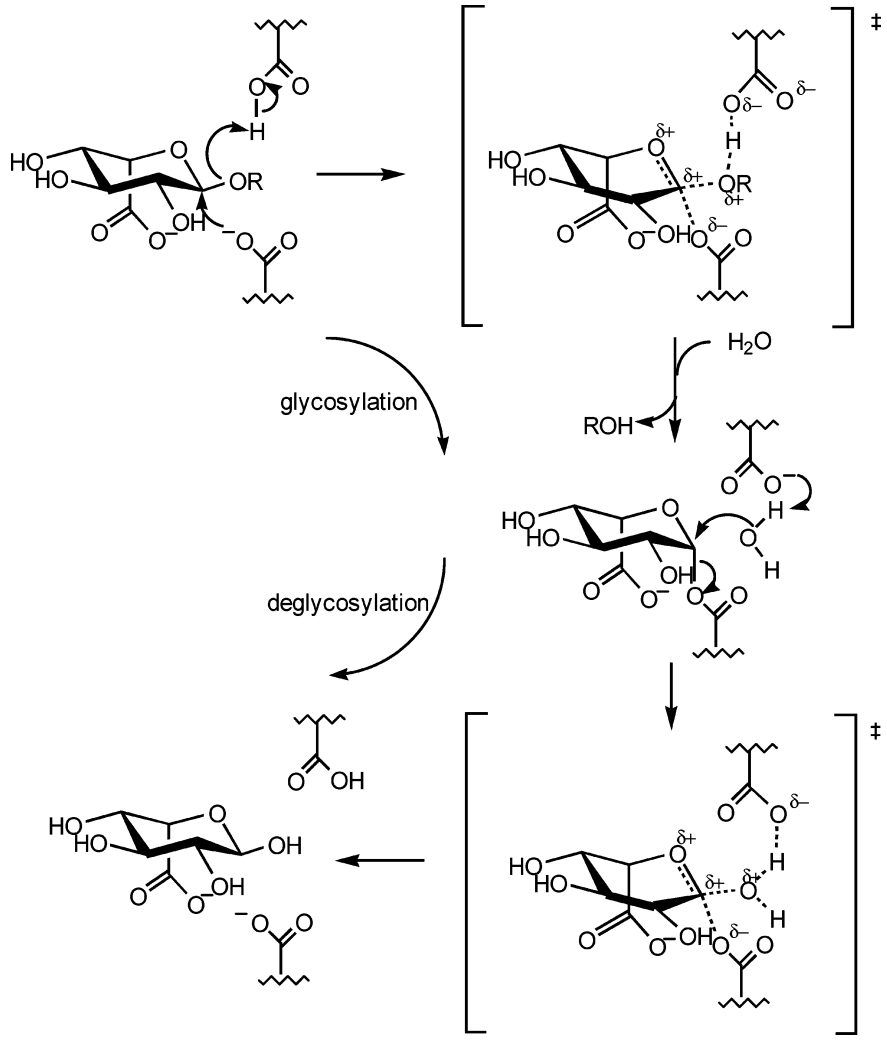
Scheme 1: Classical Covalent Mechanism for a Retaining α-L-Iduronidase



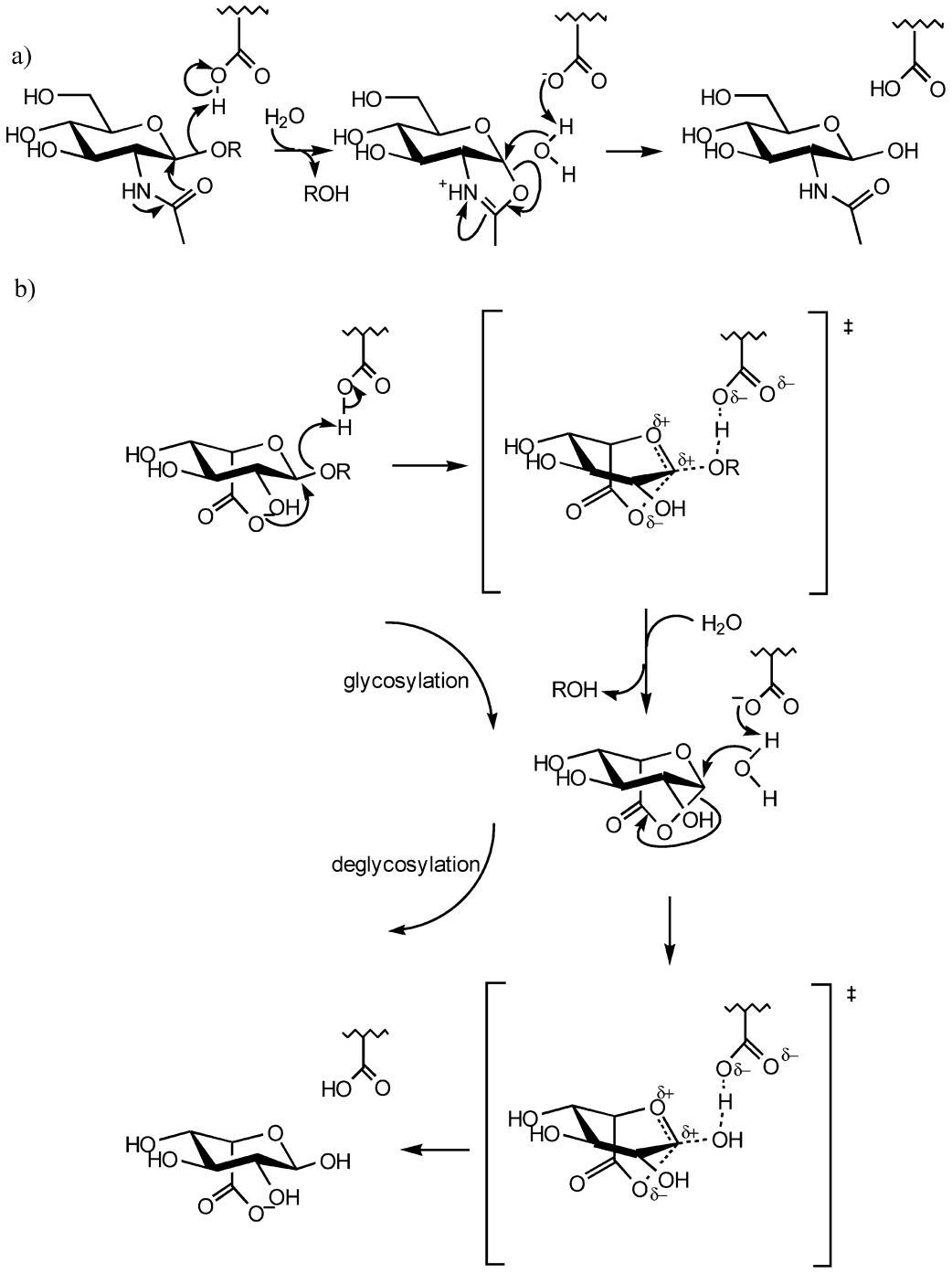
from *Thermoanaerobacterium saccharolyticum*. They found that the reaction proceeds with net retention of anomeric configuration. Because it is assigned to the same family, this implies, but does not prove, that iduronidase is also a retaining glycosidase. However, the low level of sequence similarity in the region encompassing the predicted nucleophile and the substantial difference in substrate structure bring this hypothesis into question.

Retaining glycosidases employ a two-step catalytic mechanism, as shown in Scheme 1. In the first step, a nucleophile in the enzyme active site (an aspartate or glutamate residue) attacks the anomeric center, displacing the aglycone and forming a covalent glycosyl−enzyme intermediate. In the second step, water attacks the anomeric center, displacing the enzyme nucleophile, regenerating the enzyme and producing the free sugar. A second conserved Asp or Glu residue acts as a general acid catalyst in the first step of hydrolysis, protonating the leaving aglycone, and as a general base catalyst in the second step, deprotonating the water, thus enhancing its nucleophilicity. Both steps proceed via oxocarbenium ion-like transition states.

Although most retaining glycosidases use an enzyme active site nucleophile, there are a few exceptions. For example, *N*-acetyl-β-hexosaminidases from family 20, along with family 18 chitinases, employ an anchimeric assistance mechanism in which the C2 acetamido functionality acts as the nucleophile, forming an oxazoline intermediate (*9*), which then undergoes hydrolysis (Scheme 2a). The iduronidase substrate also contains a potential nucleophile: the C6 carboxylate of iduronic acid. The presence of this functionality and the poor sequence alignment around the proposed nucleophiles of iduronidases and xylosidases make an anchimeric assistance mechanism, in which a transient lactone intermediate is formed, an alternate possibility for the mechanism of this enzyme (Scheme 2b). To distinguish between these possibilities, it would be useful to trap the intermediate that is formed and determine whether it is a glycosyl−enzyme or a lactone species.

Among the best tools for achieving this end are activated 2-deoxy-2-fluoro- and 5-fluoroglycosides. The substitution of fluorine for hydrogen or hydroxyl adjacent to either the anomeric center or the endocyclic oxygen inductively destabilizes the positively charged transition states of the retaining mechanism, thereby slowing both the glycosylation and deglycosylation steps. However, placing a very good leaving group (fluoride or 2,4-dinitrophenol) at the anomeric center accelerates the glycosylation step, without affecting the rate of the deglycosylation step. The net result is accumulation of the reaction intermediate. In this paper, we describe the use of such approaches to trap the intermediate formed on IDUA and to subsequently identify the catalytic nucleophile.

Scheme 2: (a) Neighboring Group Participation Mechanism for a Retaining Hexosaminidase and (b) Possible Participation Mechanism for Human α-L-Iduronidase



MATERIALS AND METHODS

Materials

Buffer chemicals and reagents for kinetic measurements were obtained from BDH, Boehringer Mannheim, or Sigma-Aldrich. All kinetic studies were carried out on a UNICAM 8700 UV−visible spectrophotometer equipped with a circulating water bath. Quartz or plastic cuvettes with a path length of 1 cm were used. A continuous spectrophotometric assay based on the hydrolysis of an appropriate nitrophenyl glycoside was used to monitor enzyme activity by measurement of the rate of nitrophenolate release upon hydrolysis. Enzyme concentrations and reaction times were chosen so that less than 10% of the total substrate was hydrolyzed. Human α-L-iduronidase (IDUA) was purified as described previously and stored in pH 7.0 phosphate buffer containing 0.5 M NaCl (*10*).

Determination of the Stereochemical Course of Hydrolysis

For the ^{1}H NMR-monitored determination of reaction stereochemistry, the assay buffer consisted of 20 mM 3,3-dimethylglutarate buffer, made up in D_2O. The desired pD of 4.5 was obtained by titration such that the reading on the pH meter was 4.1 (based on the equation pD = pH + 0.4, where pH is the reading on a pH meter). MUI (1 mg) was dissolved in a portion of the deuterated buffer, and the

solution was repeatedly lyophilized from 99.96% D_2O. The final lyophilization was from 99.996% D_2O, into which the residue was also redissolved. The enzyme was transferred to the deuterated buffer using a 50 kDa molecular mass cutoff centrifugal concentrator (Amicon Microcon-50). The hydrolysis experiments were performed at 25 °C using a Varian UNITY spectrometer, operating at 500 MHz. A spectrum of the substrate was first obtained, and following enzyme addition, spectra were obtained at approximately 1 min intervals.

Substrate methanolysis was first tested on a small scale. The products were separated by TLC on aluminum-backed silica plates, using a 3:1:1 butanol/acetic acid/water mixture. The plates were dried and visualized first with UV light, and then by staining with ammonium molybdate. For the NMR-scale reaction, the reaction was carried out in 20 mM 3,3-dimethylglutarate (pH 4.5) containing 3.0 M methanol, 1 mM MUI, and 0.016 μg iduronidase in a total volume of 600 μL. The reaction mixture was incubated at 37 °C for 60 min, and the enzyme was removed by centrifugation through a 50 kDa cutoff centrifugal concentrator (Amicon Microcon-50). The filtrate was lyophilized and redissolved in 99.96% D_2O several times. The final lyophilization was done from 99.996% D_2O, into which the residue was redissolved. The ^1H NMR spectrum of the resulting solution was obtained on a Bruker WH-400 spectrometer at 400 MHz at room temperature.

Sequence Alignments

Sequence alignments for the family 39 members were performed using the Clustal alignment program (found at http://www2.ebi.ac.uk/clustalw/), sponsored by the European Bioinformatics Institute. Each of the four available matrices (id, pam, blosum, and md) was tested at both the highest and lowest stringencies. The id matrix, at the lowest stringency settings, was used to generate Figure 3. The md matrix was not successful at aligning the sequences, and only reported error messages.

Kinetic Studies with 5FIdoAF

When tested as an inactivator, 5FIdoAF (0.2 and 1 mM) was incubated in buffer A [100 mM 3,3-dimethylglutarate (pH 4.5) containing 0.1% BSA] with 0.075 μg of iduronidase (total volume of 100 μL) at 37 °C. Residual enzyme activity was tested periodically by diluting 10 μL aliquots of the inactivation mix into 90 μL of substrate mix (containing 50 mM MUI in buffer A). This effectively stops the inactivation both by dilution of the inhibitor and by competition with an excess of substrate. Five minutes following the addition of the aliquot of the inhibitor mix to the substrate mix, an 80 μL aliquot of the assay was diluted into 1.4 mL of stop buffer [100 mM glycine carbonate (pH 10.7)], and the fluorescence was measured.

To test 5FIdoAF as a substrate, fluoride release was monitored using an Orion 96-09 combination fluoride ion-selective electrode fitted to a signal amplifier box, which was connected to a personal computer. Data were collected using Logger Pro (Vernier Software Inc.). All measurements were taken at 37 °C. Buffer A containing 1 mM 5FIdoAF was incubated at 37 °C for 10 min, for monitoring fluoride release to determine the rate of spontaneous hydrolysis. Iduronidase, which had been preincubated at 37 °C, was added, and fluoride release was monitored as a function of time. Kinetic parameters determined using this method were corrected to account for the ultimate release of two fluoride ions for each enzyme-catalyzed hydrolysis event.

Kinetic Studies with 2FIdoAF

The enzyme was incubated with various concentrations of the inhibitor. Aliquots (5 or 10 μL) were removed at different time intervals and diluted into assay cells containing a large volume (700 μL) of *p*-nitrophenyl α-L-iduronate substrate (at saturating concentrations, 0.35 mM = $7K_m$). The residual enzymatic activity was determined from the rate of hydrolysis of substrate, which is directly proportional to the amount of active enzyme.

Determination of Apparent K_i' Values

Apparent K_i' values for inhibitors were determined by assaying the appropriate enzyme in the presence of substrate and various concentrations of inhibitors. Initially, a V_m and K_m determination for this particular dilution of enzyme was performed. Then the same amount of enzyme was added to cells each containing the same substrate concentration ($\sim K_m$) and varying inhibitor concentrations. The steady state enzymatic rates were determined by monitoring nitrophenolate or methylumbelliferone release. A plot of $1/v$ versus inhibitor concentration intersects a line given by $1/V_m$ at $-K_i$.

Labeling and Mass Spectrometric Analysis

5FIdoAF. Iduronidase (30 μg) was transferred into sodium acetate buffer (100 mM, pH 4.5) using a 50 kDa molecular mass cutoff centrifugal concentrator (Amicon Microcon-50). 5FIdoAF was added to a concentration of 12.5 mM, and the mixture was incubated at room temperature for 30 s. The pH was brought to approximately 2 by the addition of 4 μL of phosphate buffer (pH 1.68, 2 M), and pepsin (1.5 μg) was added immediately. After 1 h at 37 °C, the digestion mixture was frozen. An unlabeled control was also established using 10 μg of iduronidase and 0.5 μg of pepsin in the absence of 5FIdoAF.

The peptide fragments were purified by reverse-phase high-pressure liquid chromatography on an Ultrafast microprotein analyzer (Michrom BioResources Inc., Pleasanton, CA) which was directly interfaced with the mass spectrometer [PE-Sciex API 300 triple-quadrupole instrument, equipped with an Ionspray ion source (Sciex, Thornhill, ON)]. The pepsin digests were loaded directly onto a C-18 column (Reliasil, 1 mm × 150 mm), which had been equilibrated with solvent A [0.05% trifluoroacetic acid (TFA) and 2% acetonitrile in water]. The peptides were eluted with a 60 min gradient to 100% solvent B (0.045% TFA and 80% acetonitrile in water), followed by 100% solvent B for 2 min. The solvents were pumped at a constant flow rate of 50 μL/min.

In the single-quadrupole mode (LC−MS), the quadrupole was scanned over an *m*/*z* range of 400−1800 Da, with a step size of 1.5 Da, and a dwell time of 0.5 ms. The orifice energy was 45 V, and the ion source voltage was 5.5 kV. In the tandem MS daughter-ion scan mode, the spectrum was obtained by selectively introducing the parent ion (*m*/*z* 893) from the first quadrupole (Q1) into the collision cell (Q2)

and then observing the product ions in the third quadrupole. The scan range for Q3 was 50−1806 Da, the step size 0.5 Da, and the dwell time 1 ms. The ion source voltage was 5 kV, and the orifice energy was 45 V; the Q0 voltage was −10 V, and the IQ2 voltage was −48 V. To increase the extent of fragmentation of the parent ion, the voltage difference between Q2 and Q0 was altered. Aminolysis of the labeled peptide was carried out by the addition of 5 μL of concentrated ammonium hydroxide to 15 μL of peptide sample, incubating for 15 min at 50 °C, neutralizing with 50% TFA, and then analyzing by ESIMS as described above.

2FIdoAF. The protocol for the 5FIdoAF labeling and analysis was followed, with the following exceptions. IDUA (40 μL, 2.7 mg/mL) was incubated with 2FIdoAF (3 μL of a 200 mM stock) at 37 °C for 15 min in 100 mM 3,3-dimethylglutarate (pH 4.5). This mixture was immediately subjected to peptic digestion. α-Iduronidase (10 μL of native or 43 μL of 2F-iduronyl−enzyme, 2.7 mg/mL) was mixed with pepsin [7 or 30 μL, respectively, 0.3 mg/mL in 2.1 M sodium phosphate buffer (pH 1.6)] and 2.1 M sodium phosphate buffer (pH 1.6) (7 or 30 μL, respectively).

Mass spectrometric conditions were as described for the 5FIdoAF labeling, with the following modifications. In the single-quadrupole mode (LC−MS), the quadrupole mass analyzer was scanned over a mass-to-charge ratio (*m*/*z*) range of 300−2200 Da with a step size of 0.5 Da and a dwell time of 1.0 ms per step. The ion source voltage (ISV) was set at 5.5 kV, and the orifice energy (OR) was 45 V. In the tandem MS daughter-ion scan mode, the spectrum was obtained by selectively introducing the parent ion (*m*/*z* 885) 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* 885. The Q3 scan range was *m*/*z* 50−1100. The step size was 0.5. The dwell time was 1.0 ms. The ion source voltage (ISV) was 5 kV. The orifice energy (OR) was 45 V. The Q0 voltage was −10 V, and the IQ2 voltage was −50 V.

Synthesis of 5-Fluoro-α-L-idopyranosyluronic Acid Fluoride

(5-Fluoro-2,3,4-tri-O-acetyl-α-L-idopyranosyl fluoride)uronic Acid. Phenacyl (2,3,4-tri-*O*-acetyl-5-fluoro-α-L-idopyranosyl fluoride)uronate (*11*) (0.086 g, 0.188 mmol) was dissolved in a 4:1 methanol/water mixture (5 mL). To this solution was added a spatula tip of 10% Pd/C. The mixture was stirred under an atmosphere of hydrogen gas at room temperature for 1 h. The suspension was then filtered through a plug of Celite 545 and the filtrate concentrated *in vacuo*. The crude material was purified by flash chromatography using a 27:2:1 ethyl acetate/methanol/water mixture to yield a gum (0.057 g, 90%): ^{1}H NMR (CD_3OD, 400 MHz) δ 5.83 (dd, 1 H, $J_{1,2}$ = 2.8 Hz, $J_{1,F}$ = 52.4 Hz, H-1), 5.58 (t, 1 H, $J_{3,2}$ = 6.8 Hz, $J_{3,4}$ = 8.8 Hz, H-3), 5.49 (dd, 1 H, $J_{4,3}$ = 8.8 Hz, $J_{4,F5}$ = 11.1 Hz, H-4), 5.24 (ddd, 1 H, $J_{2,1}$ = 2.8 Hz, $J_{2,3}$ = 6.8 Hz, $J_{2,F1}$ = 12.3 Hz, H-2), 2.00−2.10 (3 s, 9 H, OAc).

(5-Fluoro-α-L-idopyranosyl fluoride)uronic Acid. (5-Fluoro-2,3,4-tri-*O*-acetyl-α-L-idopyranosyl fluoride)uronic acid (0.249 g, 0.732 mmol) was dissolved in 10 mL of dry methanol and cooled to 0 °C. Anhydrous ammonia was bubbled through the solution until it was saturated. The solution was allowed to warm to room temperature and stirred for 30 min. The solution was then concentrated *in vacuo*. The crude material was purified by flash chromatography using a 17:2:1 ethyl acetate/methanol/water mixture to yield a gum. This was then dissolved in minimal ddH_2O and eluted through a column of Bio-Rad AG 1X-8 resin (H^+ form) using ddH_2O as the eluant. Fractions obtained were lyophilized to yield a white powder (0.133 g, 85%): ^{1}H NMR (D_2O, 400 MHz) δ 5.70 (ddd, 1 H, $J_{1,2}$ = 4.0 Hz, $J_{1,F1}$ = 55.0 Hz, $J_{1,F5}$ = 1.6 Hz, H-1), 3.98 (ddd, 1 H, $J_{4,2}$ = 1.0 Hz, $J_{4,3}$ = 7.5 Hz, $J_{4,F5}$ = 13.2 Hz, H-4), 3.92 (m, 1 H, H-2), 3.82 (t, 1 H, H-3); ^{19}F NMR (D_2O, 188 MHz) δ −26.09 (t, 1 F, $J_{F5,H4}$ = 13.2 Hz, $J_{F5,F1}$ = 12.7 Hz, F-5), −48.59 (dt, 1 F, $J_{F1,H1}$ = 55.0 Hz, $J_{F1,H2}$ = 9.1 Hz, $J_{F1,F5}$ = 12.7 Hz, F-1). Anal. Calcd for $C_6H_9O_3F_3 \cdot H_2O$: C, 31.04; H, 4.34. Found: C, 31.01; H, 4.32.

Synthesis of 2-Deoxy-2-fluoro-α-L-idopyranosyluronic Acid Fluoride

Phenacyl (3,4-Di-O-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride)uronate. Phenacyl (2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride)uronate (1.49 g, 4.71 mmol) (*12*) was dissolved in dry dichloromethane (15 mL). To the solution was added acetyl chloride (1.0 mL, 14.1 mmol, 3 equiv) followed by triethylamine (1.4 mL, 9.89 mmol, 2.1 equiv). After being stirred at room temperature for 3 h, the solution was washed with 1 N HCl followed by saturated sodium bicarbonate, dried over magnesium sulfate, and concentrated *in vacuo*. Silica gel chromatography (2:1 petroleum ether/ethyl acetate mixture) yielded a white foam (1.51 g, 80%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83−7.88 (m, 2 H, H-2′, H-6′), 7.57−7.64 (m, 1 H, H-4′), 7.43−7.48 (m, 2 H, H-3′, H-5′), 5.55 (dt, 1 H, $J_{1,2}$ = 5.3 Hz, $J_{1,F1}$ = 51.2 Hz, H-1), 5.45 (t, $J_{4,3}$ = 9.1 Hz, $J_{4,5}$ = 8.5 Hz, H-4), 5.34−5.42 (m, 3 H, 2× OCH-*H*, H-3), 4.58 (dddd, 1 H, $J_{2,1}$ = 5.3 Hz, $J_{2,F1}$ = 10.3 Hz, $J_{2,3}$ = 7.2 Hz, $J_{2,F2}$ = 49.2 Hz, H-2), 4.48 (d, 1 H, $J_{5,4}$ = 8.5 Hz, H-5), 2.10−2.30 [2 s, 6 H, 2× (CO)CH_3]; ^{19}F NMR (188 MHz, $CDCl_3$) δ −61.92 (dt, 1 F, $J_{F,H1}$ = 51.2 Hz, $J_{F,H2}$ = 10.3 Hz, $J_{F1,F2}$ = 14.2 Hz, F-1), −123.65 (m, 1 F, F-2).

Phenacyl (5-Bromo-3,4-di-O-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride)uronate. Phenacyl (3,4-di-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride)uronate (1.51 g, 3.77 mmol) was dissolved in dry CCl_4 (40 mL), recrystallized *N*-bromosuccinimide (1.00 g, 1.5 equiv) added, and the mixture irradiated with two 200 W light bulbs. After 45 min, the resulting orange mixture was filtered through glass wool and the solvent evaporated. The crude material was purified by flash chromatography using a 1:3 ethyl acetate: petroleum ether mixture to yield a gum (0.93 g, 51%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83−7.90 (m, 2 H, H-2′, H-6′), 7.58−7.64 (m, 1 H, H-4′), 7.43−7.50 (m, 2 H, H-3′, H-5′), 5.84 (ddd, 1 H, $J_{1,2}$ = 7.2 Hz, $J_{1,F1}$ = 51.3 Hz, $J_{1,F2}$ = 4.7 Hz, H-1), 5.65 (dd, $J_{3,2}$ = 9.3 Hz, $J_{3,4}$ = 9.3 Hz, $J_{3,F2}$ = 23.0 Hz, H-3), 5.39−5.49 (m, 2 H, 2× OCH-*H*, H-4), 4.63 (dddd, 1 H, $J_{2,1}$ = 7.2 Hz, $J_{2,F1}$ = 13.9 Hz, $J_{2,3}$ = 9.3 Hz, $J_{2,F2}$ = 50.6 Hz, H-2), 2.09 [s, 6 H, 2× (CO)CH_3]; ^{19}F NMR (188 MHz, $CDCl_3$) δ −75.01 (dt, 1 F, $J_{F,H1}$ = 51.3 Hz, $J_{F,H2}$ = 13.9 Hz, $J_{F1,F2}$ = 15.1 Hz, F-1), −118.35 (m, 1 F, F-2).

Phenacyl (3,4-Di-O-acetyl-2-deoxy-2-fluoro-α-L-idopyranosyl fluoride)uronate. Phenacyl (5-bromo-3,4-di-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride)uronate **11**

(0.93 g, 1.9 mmol) was dissolved in dry toluene (20 mL), tributyltin hydride added (1.0 mL, 3.9 mmol, 2 equiv), and the solution boiled at reflux for 2 h. The solvent was then removed *in vacuo*, 20 mL of acetonitrile added, and the mixture extracted with petroleum ether (4 × 20 mL). The acetonitrile layer was concentrated *in vacuo*, and the residue was purified by flash chromatography using a 1:3 ethyl acetate/petroleum ether mixture to yield white needles (0.235 g, 30%). Also, the *gluco* epimer was obtained (0.226 g, 29%). Both products were rechromatographed to remove residual tributyltin compounds. The *ido* product was recrystallized from an ethyl acetate/petroleum ether mixture to yield white needles that were suitable for X-ray crystallography: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.83−7.89 (m, 2 H, H-2′, H-6′), 7.57−7.62 (m, 1 H, H-4′), 7.45−7.52 (m, 2 H, H-3′, H-5′), 5.87 (dd, 1 H, $J_{1,F2}$ = 7.2 Hz, $J_{1,F1}$ = 47.4 Hz, H-1), 5.53 (d, 1 H, OCH-*H*), 5.34 (d, 1 H, OCH-*H*), 5.33 (m, 1 H, H-4), 5.20−5.28 (m, 1 H, H-3), 5.11 (d, 1 H, $J_{5,4}$ = 1.7 Hz, H-5), 4.44−4.64 (m, 1 H, H-2), 2.20 [s, 3 H, $(CO)CH_3$], 2.14 [s, 1 H, $(CO)CH_3$]; ^{19}F NMR (282 MHz, $CDCl_3$) δ −62.89 (dd, 1 F, $J_{F,H1}$ = 47.4 Hz, $J_{F1,F2}$ = 14.0 Hz, F-1), −118.08 (dddd, 1 F, $J_{F2,H1}$ = 7.2 Hz, $J_{F2,H3}$ = 9.6 Hz, $J_{F1,F2}$ = 14.0 Hz, $J_{F2,H2}$ = 42.8 Hz, F-2).

3,4-Di-O-acetyl-2-deoxy-2-fluoro-α-L-idopyranosiduronic Acid Fluoride. Phenacyl (3,4-di-*O*-acetyl-2-deoxy-2-fluoro-α-L-idopyranosyl fluoride)uronate (0.216 g, 0.540 mmol) was dissolved in a 1:1 ethyl acetate/methanol mixture (8 mL), 10% Pd-C (0.060 g, 0.1 equiv) added, and the suspension stirred at room temperature under an atmosphere of hydrogen gas for 3 h. The suspension was then filtered through a plug of Celite 545 and the filtrate concentrated *in vacuo*. The crude material was purified by flash chromatography using initially ethyl acetate, followed by a 1:1 ethyl acetate/methanol mixture, to yield a white solid (0.134 g, 88%): ^{1}H NMR (400 MHz, CD_3OD) δ 5.58 (dd, 1 H, $J_{1,F2}$ = 8.3 Hz, $J_{1,F1}$ = 48.6 Hz, H-1), 5.25 (s, 1 H, H-4), 5.21 (d, 1 H, $J_{3,F2}$ = 10.5 Hz, H-3), 4.70 (s, 1 H, H-5), 4.60 (d, 1 H, $J_{2,F2}$ = 43.3 Hz, H-2), 2.12 [s, 3 H, $(CO)CH_3$], 2.03 [s, 1 H, $(CO)CH_3$].

2-Deoxy-2-fluoro-α-L-idopyranosyluronic Acid Fluoride. 3,4-Di-*O*-acetyl-2-deoxy-2-fluoro-α-L-idopyranosyluronic acid fluoride **13** (0.134 g, 0.48 mmol) was dissolved in dry methanol (10 mL) and cooled to 0 °C under argon. Anhydrous ammonia was then bubbled through the solution until it was saturated. After the mixture had been stirred at room temperature for 2 h, the reaction was still incomplete so 10 drops of a 0.1 M NaOMe/MeOH mixture was added and the reaction mixture stirred for a further 30 min. Silica gel (230−400 mesh) was added to the solution, the solvent removed *in vacuo*, and the silica gel layered atop a flash column. Ethyl acetate was used as the initial eluant, followed by a 7:2:1 ethyl acetate/methanol/water mixture, and finally a 5:2:1 ethyl acetate/methanol/water mixture. Fractions containing the desired product were concentrated, redissolved in ddH_2O, filtered through a 0.22 μm filter, and passed through a column of Bio-Rad AG 50WX-8 resin (H^+ form) using ddH_2O as the eluant. The fractions that were obtained were lyophilized to yield an impure white foam (0.070 g, 74%) which was dissolved in dimethylformamide (0.5 mL) and ethyl acetate (1 mL). To the stirring solution was added phenacyl bromide (0.141 g, 2 equiv), followed by a solution of triethylamine (59 μL, 1.2 equiv) predissolved in ethyl acetate (2 mL). After being stirred overnight, the mixture was washed with water, dried over magnesium sulfate, and concentrated *in vacuo*. The crude material was purified by flash chromatography using a 1:1 ethyl acetate/petroleum ether mixture to yield a white solid (0.037 g, 33%). The ester was then dissolved in ethyl acetate (5 mL) and methanol (5 mL), 10% Pd-C (0.020 g, 0.18 equiv) added, and the suspension stirred at room temperature under an atmosphere of hydrogen gas for 2.5 h. The suspension was then filtered through a plug of Celite 545 and the filtrate concentrated *in vacuo*. The crude material was purified by flash chromatography using initially ethyl acetate, followed by a 1:1 ethyl acetate/methanol mixture, then a 1:2 ethyl acetate/methanol mixture, and finally a 1:3 ethyl acetate/methanol mixture. Fractions containing the desired product were concentrated, redissolved in ddH_2O, filtered through a 0.22 μm filter, and passed through a column of Bio-Rad AG 50WX-8 resin (H^+ form) using ddH_2O as the eluant. The fractions that were obtained were lyophilized to yield a white foam (0.019 g, 95%): ^{1}H NMR (300 MHz, D_2O) δ 5.75 (dd, 1 H, $J_{1,F2}$ = 8.6 Hz, $J_{1,F1}$ = 48.0 Hz, H-1), 4.78 (s, 1 H, H-5), 4.52 (d, 1 H, $J_{2,F2}$ = 43.0 Hz, H-2), 4.00−4.10 (m, 2 H, H-3, H-4); ^{19}F NMR (282 MHz, D_2O) δ −61.50 (dd, 1 F, $J_{F,H1}$ = 48.0 Hz, $J_{F1,F2}$ = 10.0 Hz, F-1), −117.22 (m, 1 F, F-2). Anal. Calcd for $C_6H_8O_5F_2 \cdot {}^1/_2H_2O$: C, 34.79; H, 4.38. Found: C, 35.16; H, 4.32.

RESULTS AND DISCUSSION

Stereochemical Outcome of Iduronidase Catalysis. The stereochemical outcome of glycosidase-catalyzed reactions is generally best determined by direct ^{1}H NMR analysis of the enzyme-catalyzed reaction. Real time monitoring of the chemical shift of the anomeric proton and of its coupling constant with H2 allows the determination of the stereochemistry of the initial product formed in the reaction. A limiting factor in such an approach is the rate of mutarotation of the product sugar. However, at neutral pH and with sufficient enzyme, the desired result can generally be obtained. In contrast to many other glycosidases investigated using this technique, the pH optimum of the IDUA-catalyzed reaction is quite low [∼4.5 (*13*)]. At such a low pH, the acid-catalyzed mutarotation reaction is sufficiently rapid to minimize the accumulation of the initial product, thus making its characterization by ^{1}H NMR impossible, despite extensive efforts to accumulate data as quickly as possible. Even when data were collected within minutes of enzyme addition, the anomeric protons of both the α- and β-anomers of the product were visible at similar intensity, and by 5 min, in addition to the α- and β-pyranose products, the α- and β-furanose products also appeared. The substrate was completely consumed after approximately 20 min, at which time an equilibrium mixture of the four aforementioned products also existed (Figure 1). Such rapid mutarotation of IdoA is not too surprising given that uronic acids undergo relatively rapid anomeric interconversions, presumably via intramolecular general acid catalysis, and given the relatively strained nature of α-L-iduronic acid. The appearance of the furanose forms of IdoA after only 5 min confirms that reaction via the acyclic form of the sugar has occurred (Scheme 3). Attempts to carry out the reaction at a higher pH were unsuccessful. Success was ultimately achieved by carrying out a methanolysis reaction through inclusion of

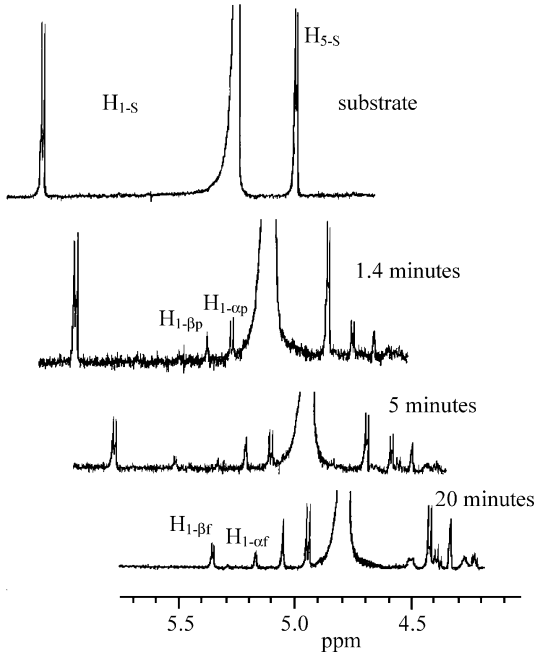


FIGURE 1: ^{1}H NMR analysis of iduronidase-catalyzed hydrolysis of methylumbelliferyl iduronide. Spectra were recorded at the indicated times. H_{1-S} is substrate H1, H_{5-S} substrate H5, $H_{1-\alpha p}$ H1 of α-idopyranuronic acid, $H_{1-\beta p}$ H1 of β-idopyranuronic acid, $H_{1-\alpha f}$ H1 of α-idofuranuronic acid, and $H_{1-\beta f}$ H1 of β-idofuranuronic acid.

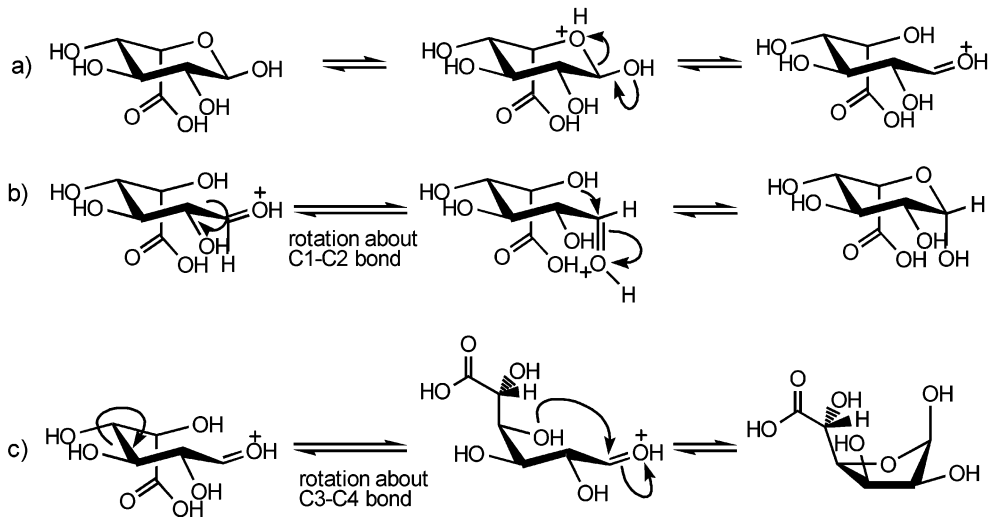
methanol in the buffer, since the resulting methyl glycoside does not undergo mutarotation. Fortunately, IDUA, being a robust lysosomal enzyme, is quite stable in the presence of molar concentrations of methanol.

Methanolysis reactions were initially monitored by thin-layer chromatography. The substrate [methylumbelliferyl α-L-iduronide (MUI)] was incubated with IDUA in the presence of varying concentrations of methanol (from 0 to 6 M). In the absence of methanol, the reaction goes nearly to completion, leaving a small amount of intact substrate (R_f = 0.39) along with 4-methylumbelliferone (R_f = 0.79) and free iduronic acid (R_f = 0.21). The addition of methanol to the reaction mixture caused a fourth spot to appear (R_f = 0.27), which was only very slightly less polar than the free iduronic acid. As with the free iduronic acid, this spot was neither UV active nor fluorescent, as expected for the methyl glycoside of iduronic acid. At higher concentrations of methanol, the intensity of this spot increased, with a concurrent decrease in intensity of the spot from free iduronic acid. At 3 M methanol, the methanolysis product appeared to be approximately 50% of the product formed, but when the methanol concentration was further increased to 6 M, only a moderate further increase in the proportion of methanolysis product was observed.

Using 3 M methanol, the above experiment was repeated on a larger scale, and the products were analyzed by ^{1}H NMR (Figure 2). Shown in gray is the spectrum from the mixture of the α/β-L-idopyranuronic acid and α/β-L-idofuranuronic acid. The anomeric protons clearly correspond with the anomeric protons observed in the earlier "real time" hydrolysis experiment. However, clearly, an additional product was observed, in approximately 50% yield (shown in black). The chemical shifts and coupling constants of this product are in agreement with those published for the sodium salt of methyl α-L-idopyranosiduronic acid (*14*). None of the β-glycoside was observed. The iduronidase is therefore a retaining enzyme, consistent with its assignment to glycosidase family 39.

Identification of the Nucleophile via Sequence Alignments. As shown in Figure 3a, alignment of the sequences of selected family 39 enzymes around the proposed acid/base residue shows a very high degree of similarity, and there is a very high probability that the prediction of E178 as the catalytic acid/base residue of human iduronidase is correct. The case is quite different, however, for the sequence alignment surrounding the proposed catalytic nucleophiles (Figure 3b). Upon examination of the amino acid sequences in this section, it appears as though family 39 could be broken down into two subfamilies: the xylosidases and the iduronidases. In fact, only one of the four sequence alignment matrices within the ClustalW sequence alignment program will align the xylosidase nucleophiles with the iduronidase nucleophiles proposed by Henrissat (*15*) (Figure 3b), and

Scheme 3: Mechanisms for Mutarotation and Isomerization of Iduronic Acid[a]



[a] This scheme is illustrative of the bond breaking and forming steps, but is not intended to indicate a specific acid-catalyzed process. Indeed, some form of intramolecular general acid-catalyzed process is most probable.

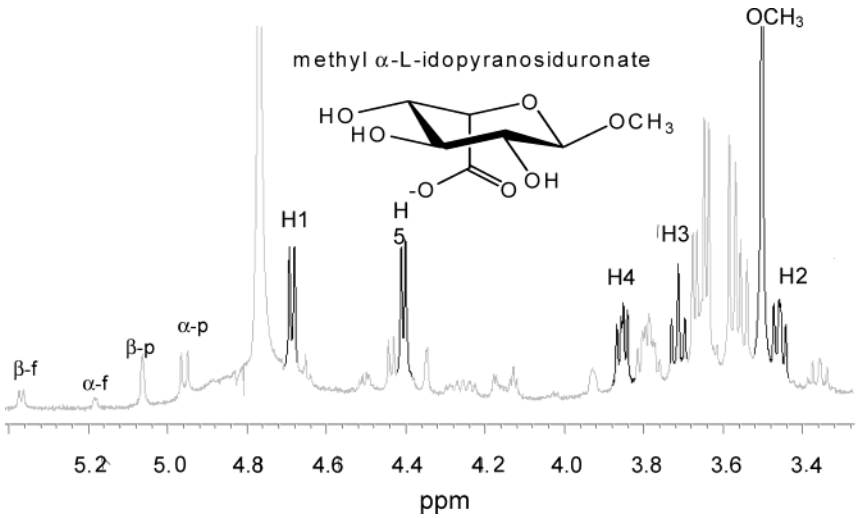


FIGURE 2: ^{1}H NMR analysis of iduronidase-catalyzed cleavage of methylumbelliferyl iduronide in the presence of 3 M methanol.

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a)
Thermoanaerobacterium saccharolyticum (xyl)      139 HHFIS RYGIE EVLKW PFEIW NEPNL KEFWK DADEK EYFKL YKVTA
Thermoanaerobacterium sp. (xyl)                  139 SHFIS RYGID EVAKW PFEIW NEPNL KEFWK DADEK EYFKL YKITA
Caldicellulosiruptor saccharoylticus (xyl-D)     145 KHFID RYGEK EVVQW PFEIW NEPNL NVFWK DANQA EYFKL YEVTA
Bacillus stearothermophilus (xyl)                139 SHFIE RYGIE EVRTW LFEVW NEPNL VNFWK DANKQ EYFKL YEVTA
Caldicellulosiruptor saccharoylticus (xyl-B)     142 RHLIS RYGKN EVREW FFEVW NEPNL KDFFW AGTME EYFKL YKYAA

Canis familiaris (idua)                          160 RRYIG RYGLS YVSKW NFETW NEPDH HDFDN VTMTL QGFLN YYDAC
Mus musculus (idua)                              151 RRYIG RYGLT HVSKW NFETW NEPDH HDFDN VSMTT QGFLN YYDAC
Homo sapiens (idua)                              161 RRYIG RYGLA HVSKW NFETW NEPDH HDFDN VSMTM QGFLN YYDAC
                                                      : *  ***    *  *  ** * ***:    *         : *   *  .
                                                                                ◊
b)
Thermoanaerobacterium saccharolyticum (xyl)      266 SH--F PNLPF HITEY NTSY- -SPQN PVHDT PFNAA YIARI LSEGG
Thermoanaerobacterium sp. (xyl)                  266 SH--F PNLPF HITEY NTSY- -SPQN PVHDT PFNAA YIARI LSEGG
Caldicellulosiruptor saccharoylticus (xyl-D)     272 SP--F PDLPI HITEF NSSY- -HPLC PIHDT PFNAA YLARV LSEAG
Bacillus stearothermophilus (xyl)                269 SP--F PHLPL HITEY NTSY- -SPIN PVHDT ALNAA YIARI
Caldicellulosiruptor saccharoylticus (xyl-B)     269 ----- --LPV YYTEW NNSP- -SPRD PYHDI PYDAA FIVKT IIDII

Canis familiaris (idua)                          286 LFPKF ADTPV YNDEA DPLVG WALPQ PWRAD VTYAA MVVKV VAQHQ
Mus musculus (idua)                              276 LFPEF KDTPI YNDEA DPLVG WSLPQ PWRAD VTYAA LVVKV IAQHQ
Homo sapiens  (idua)                             286 LFPKF ADTPI YNDEA DPLVG WSLPQ PWRAD VTYAA MVVKV IAQHQ
                                                       *. :  *  :          * :      **  :.:  : :
                                                             ✪
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FIGURE 3: Sequence alignments of the family 39 glycosidases in the regions flanking the predicted nucleophiles and acid/base catalysts. xyl represents xylosidase and idua iduronidase: (◇) acid/base residue and (☆) nucleophile.

this alignment requires the lowest-stringency settings within the program. The other alignment matrices place aspartate 301 or alanine 314 in this position; some even suggest that there is no catalytic nucleophile in the iduronidases, aligning the xylosidase nucleophiles with a gap in the iduronidase sequences. This raises some doubt about whether the prediction of E299 as the human iduronidase nucleophile is correct.

Trapping an Intermediate Using 5FIdoAF. To resolve this mechanistic controversy, 5-fluoro-α-L-idopyranosyluronic acid fluoride (5FIdoAF) (Figure 4a) was synthesized and tested as a potential inactivator of IDUA. The synthesis largely followed a published procedure (*11*), with the final deprotection steps involving hydrogenation of the phenacyl protecting group and deacetylation with ammonia in methanol. No time-dependent inactivation was observed when aliquots were sampled and assayed. However, the observed enzyme activity decreased with increasing concentrations of 5FIdoAF, indicating the apparent reversible inhibition by the material carried over into the assay mix, rather than time-dependent inactivation (Figure 4b). 5FIdoAF was therefore tested as a reversible competitive inhibitor of IDUA using MUI (V_{max} = 12 μmol min^{-1} mg^{-1}, K_m = 15 μM) as the substrate. Dixon plot analysis of the inhibition data (Figure 4c) revealed a K_i' value of 1.2 μM. This apparently tight binding inhibition is highly indicative of the accumulation of an enzyme-bound 5-fluoroiduronyl intermediate that turns over quickly relative to the time scale of the assay, rather than true, tight, reversible binding. This was confirmed by testing the 5FIdoAF as a substrate by monitoring fluoride release with a fluoride specific electrode, allowing the measurement of a k_{cat} value of 0.83 s^{-1}. This value is therefore the deglycosylation rate constant, and clearly indicates that reactivation of the trapped fluoroglycosyl−enzyme intermediate formed in the inactivation mixture would turn over during the assay time. Thus, the observed inhibition is that arising from the steady state accumulation of the level of intermediate formed from the low concentration of 5FIdoAF carried over into the assay mixture. A determination of K_m was not possible given the (in)sensitivity of the electrode and the low anticipated K_m (=K_i', 1.2 μM) value.

These low K_m (K_i') and k_{cat} values are consistent with accumulation of some intermediate along the reaction pathway: either the glycosylated enzyme or a tightly bound noncovalent species such as the lactone illustrated in Scheme 2b.

Labeling IDUA with 5FIdoAF. Distinction between the two mechanisms was achieved by mass spectrometric means. Unfortunately, heterogeneous glycosylation of the enzyme rendered direct detection of the modified enzyme impossible; thus, a search was initiated for a glycosylated peptide in proteolytic digests.

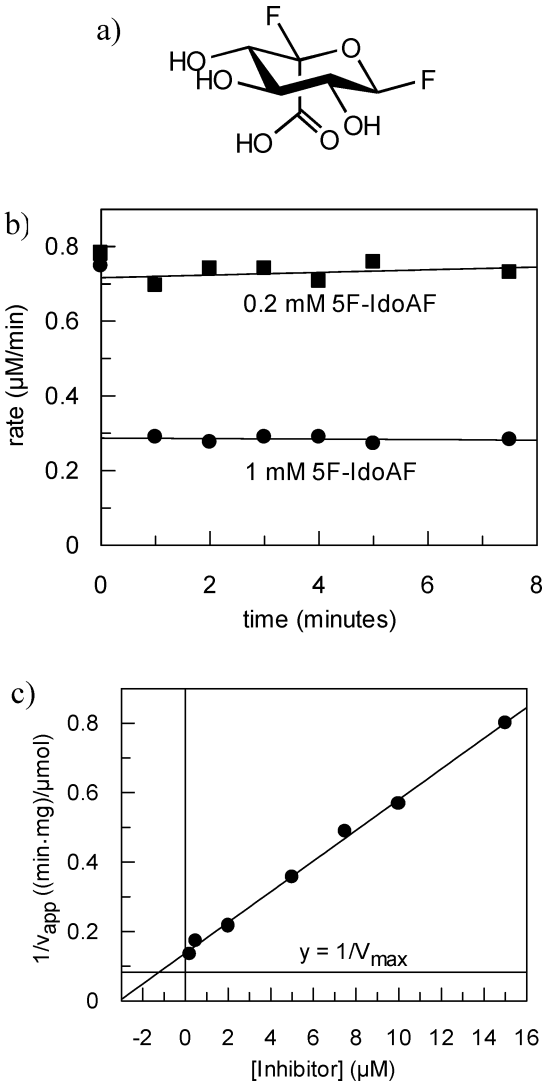

FIGURE 4: Kinetic analysis of the interaction of iduronidase with 5-fluoro-α-L-iduronyl fluoride. (a) Structure of 5-fluoro-α-L-iduronyl fluoride. (b) Time dependence of the interaction of 5-fluoro-α-L-iduronyl fluoride with iduronidase. (c) Dixon plot of the inhibition of iduronidase by 5-fluoro-α-L-iduronyl fluoride. MUI (50 μM) was used as the substrate.

IDUA was incubated with 5FIdoAF, then denatured at low pH, and digested with pepsin. Comparison of the LC−MS profile of this digest with that from an equivalently treated, but unlabeled, control sample revealed the presence of a labeled peptide having an *m*/*z* of 893 ± 1 that was found at 33.5 min in the total ion current chromatogram of the labeled peptide digest, this fragment being absent in the unlabeled digest. Treatment of this peptide with ammonium hydroxide caused its *m*/*z* to decrease by 97, corresponding to the loss of a label from a doubly charged peptide with a molecular mass of 194 g/mol, consistent with the loss of the 5-fluoroiduronyl label.

The parent peptide fragment (minus the sugar) thus has a molecular mass of 1590 Da. Seventeen candidate peptides with a mass of 1590 ± 1 Da were identified by inspecting the amino acid sequence of the enzyme and searching for all possible peptides with this mass. Of these, all but six were provisionally eliminated, either because their sequences did not contain an aspartate or a glutamate residue or because the peptides contained more than one basic residue, which would produce a triply charged species. An ester-linked intermediate is also indicated by the ammonium hydroxide cleavage. The candidate peptides were $_{122}$RENQLLPGFELMGS$_{135}$, $_{245}$TNFFTGEAGVRLDY$_{258}$, $_{291}$ADTPIYNDEADPLVG$_{305}$, $_{293}$TPIYNDEADPLVGW$_{306}$, $_{401}$LWAEVSQAGTVLDSN$_{490}$, and $_{605}$FVFSPDTGAVSGSYR$_{619}$. The peptide was then unambiguously identified by peptide sequencing via tandem MS analysis of the labeled peptide of interest (*m*/*z* 893) in the daughter-ion scan mode (Figure 5). Peaks resulting from Y″ and B ions are described in detail in Table 1. 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 $_{291}$ADTPIYNDEADPLVG$_{305}$.

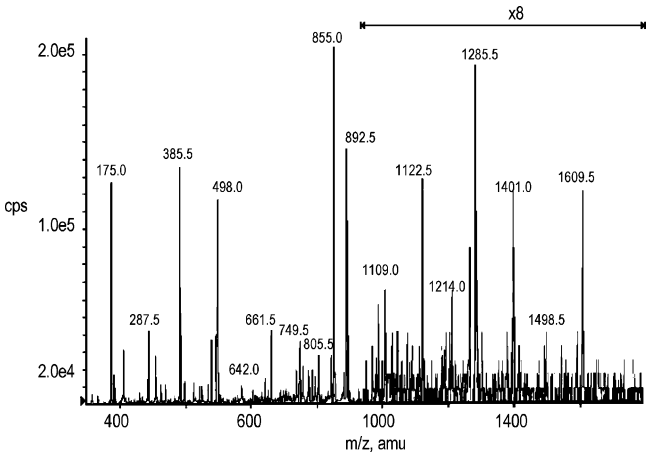

FIGURE 5: Tandem MS analysis of daughter ions from fragmentation of the 5-fluoroiduronyl-labeled peptide.

Table 1[a]

fragment (*m*/*z*)	ion type	corresponding sequence
855[d]	B	ADTPIYNDEADPLV*
175[s]	Y	VG
288[s]	B or Y	ADT or LVG, respectively
386[s]	B	ADTP
498[s]	B	ADTPI
661[s]	B	ADTPIY
749[d]	B	ADTPIYNDEADP*
855[d]	B	ADTPIYNDEADPLV*
892[d]	parent ion	ADTPIYNDEADPLVG*
1123[s]	Y	NDEADPLVG*
1286[s]	B	ADTPIYNDEA*
1401[s]	B	ADTPIYNDEAD*
1610[s]	B	ADTPIYNDEADPL*

[a] Asterisks denote peptide fragments containing the label. A superscript s denotes a singly charged fragment and the superscript d a doubly charged fragment.

The observed B ions containing the label were ADTPIYNDEA (*m*/*z* 1286), ADTPIYNDEAD (*m*/*z* 1401), ADTPIYNDEADP (*m*/*z* 749), ADTPIYNDEADPL (*m*/*z* 1610), and ADTPIYNDEADPLV (*m*/*z* 855). These data indicate that the label is located within the ADTPIYNDEA section of the peptide. Only one Y″ ion containing the label was observed (NDEADPLVG). Together with the B ion data, this indicates that the label had to be localized to the tetrapeptide NDEA. Alanine 300 can be ruled out, as it does not have a functional group capable of acting in a nucleophilic manner, and asparagine 297 can most likely be ruled out on the basis of the aforementioned precedent. We can

Table 2[a]

fragment (*m*/*z*)	ion type	corresponding sequence
175	Y	VG
288	B or Y	ADT or LVG, respectively
385	B or Y	ADTP or PLVG, respectively
498	B	ADTPI
500	Y	DPLVG
571	Y	ADPLVG
663	B	ADTPIY
775	B	ADTPIYN
876	**Y**	**EADPLVG***
885	parent ion (doubly charged)	ADTPIYNDEADPLVG*
890	**B**	**ADTPIYND**
993	Y	DEADPLVG*
1108	Y	NDEADPLVG*
1198	B	ADTPIYNDE*
1270	Y	YNDEADPLVG*
1383	B or Y	ADTPIYNDEAD* or IYNDEADPLVG*, respectively
1480	B or Y	ADTPIYNDEADP* or PIYNDEADPLVG*, respectively
1593	B	ADTPIYNDEADPL*

[a] Asterisks denote peptide fragments containing the label; bold type denotes peptides key in determining the location of the label.

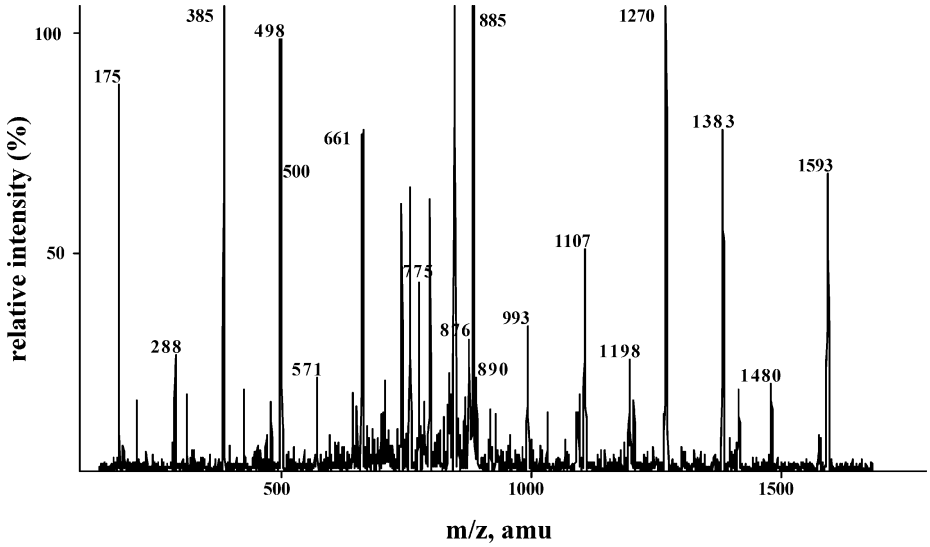

FIGURE 6: Tandem MS analysis of daughter ions from fragmentation of the 2-deoxy-2-fluoroiduronyl-labeled peptide.

therefore conclude that the label is localized to either aspartate 298 or glutamate 299.

Unfortunately, it was not possible to show conclusively whether D298 or E299 carries the label, since to do so, the labeled fragment *EADPLVG, having *m*/*z* 895, would have to be observed. As luck would have it, the intact, labeled peptide appears as a strong signal at *m*/*z* 893; thus, the peak sought (*m*/*z* 895) could quite easily be lost in the shoulder of the more intense peak of the intact, doubly charged parent peptide. Attempts to more aggressively fragment this peptide resulted in excessive noise in the data. Attention therefore turned to the use of a second label to provide additional insights.

Trapping the Intermediate with 2FIdoAF. While 5-fluoro sugar derivatives have been used in labeling both α-D- and β-D-glycosidases, 2-deoxy-2-fluoro sugars have previously been useful only for β-D-glycosidases. Because it could not be predicted whether α-L-iduronidase would act more like an α-D- or a β-D-glycosidase, we felt that the 2-fluoro-α-L-idopyranosyluronic acid fluoride (2FIdoAF) (Figure 6a) was worth pursuing as a potential labeling agent; not only would this provide independent confirmation of the identity, but the labeled peptide (EADPLVG*) should also have an ion at *m*/*z* 878, which should be well separated from the intense signal arising from the parent ion at *m*/*z* 885.

Synthesis of 2FIdoAF was achieved from a protected 2-fluoro-β-D-glucuronyl fluoride precursor *via* radical bromination at C5, reaction with tri-*n*-butyltin hydride to displace the bromide with net inversion of configuration and then deprotection, as described in Materials and Methods. 2FIdoAF was tested as a time-dependent inactivator of IDUA, and as with 5FIdoAF, no time-dependent inactivation was found. Instead, enzyme activity dropped immediately to a constant level, with the extent of inhibition varying with the concentration of 2FIdoAF. When 2FIdoAF was tested as an apparent reversible competitive inhibitor of IDUA using 4-nitrophenyl α-L-idopyranosiduronate (pNPIdoA) ($K_m = 53$ μM, $V_{max} = 4.4$ μmol min^{-1} mg^{-1}) as the substrate, a K_i' value of 4.6 μM was found.

IDUA inactivated by 2FIdoAF was then proteolytically digested and the resultant peptide mixture analyzed by LC−MS. Comparative mapping revealed a peptide of *m*/*z* 1769 present only in the labeled sample, along with an *m*/*z* 885 peptide fragment corresponding to the doubly charged

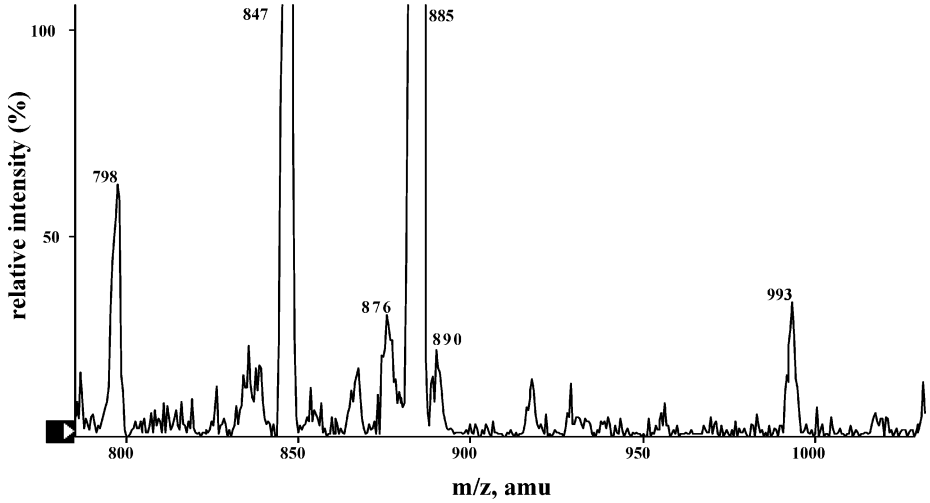

FIGURE 7: Expansion of the region in Figure 6 around *m*/*z* 900.

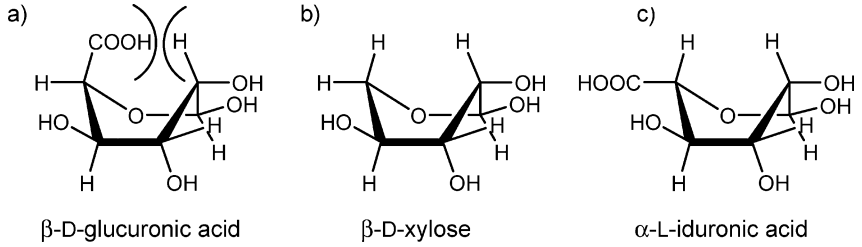

FIGURE 8: Comparison of $^{1,4}B$ conformations of β-D-glucuronic acid, β-D-xylose, and α-L-iduronic acid.

species. Given that this label has a mass of 179 Da, the peptide in question must have an ion at *m*/*z* 1590, as seen before. Sequencing by MS/MS identified the peptide as $_{291}$ADTPIYNDEADPLVG$_{305}$, a peptide identical to that isolated following labeling with 5FIdoAF (Figure 6). In this case, expansion of the region around the parent peptide revealed a fragment at *m*/*z* 876 (Figure 7), consistent, within an error of ± 2, with the expected mass for the labeled fragment EADPLVG*. Further, the observed peak at *m*/*z* 890 corresponds to the unlabeled peptide fragment ADTPIYND; no evidence for the labeled version of this peptide (*m*/*z* 1068) was found in the mass spectrum, nor was this peptide observed in digests of the 5FIdoAF-labeled enzyme. On the basis of these arguments, in conjunction with the data from the 5FIdoAF labeling, we conclude that Glu299 is, indeed, the labeled residue, and hence the catalytic nucleophile in human α-L-iduronidase. These results are consistent with those of a mutational analysis performed on human iduronidase in which the proposed acid/base (E182) and nucleophile (E299) were each, individually, mutated to alanine (*16*). No significant activity could be detected in cell extracts, or in immunoprecipitates, supporting the assignment of important roles to these residues. However, kinetic studies on purified and characterized mutants were not performed.

These results therefore demonstrate that both the xylosidases and iduronidases carry out catalysis *via* a covalent glycosyl−enzyme intermediate formed at the same location, thereby confirming the (weak) predicted alignments around the catalytic nucleophile. A likely rationale for the presence of β-D-xylosidases and α-L-iduronidases in the same family comes from a consideration of the possible sugar conformations adopted during catalysis. The crystal structures of the intermediates formed on two family 11 xylanases reveal a 2-deoxy-2-fluoroxylosyl moiety bound at the −1 subsite in the rather uncommon boat ($^{2,5}B$) conformation (*17*, *18*). Such a geometry places O5, C5, C1, and C2 in a plane, as would be required to stabilize positive charge at the anomeric center. It was therefore suggested that the transition state for these enzymes adopts a similar geometry, rather than the half-chair that is generally expected. The reason this conformation is so seldom observed, or even considered, as a reasonable transition state geometry is that most common sugars (glucose, galactose, mannose, and glucuronic acid) possess an equatorial C5 hydroxymethyl or carboxylate group. In a $^{2,5}B$ conformation, this substituent would be forced into a pseudoaxial orientation causing destabilizing flagpole interactions with the C2 hydrogen (Figure 8a). In contrast, xylose does not have a C5 substituent, and therefore, there is no such highly unfavorable interaction, allowing the xylanase substrate to readily adopt this conformation in the enzyme active site (Figure 8b). While no crystal structures are yet available for any of the family 39 enzymes, it is quite possible that the xylosidases in this family also carry out catalysis via a $^{2,5}B$ conformation since they have no bulky C5 substituent. Interestingly, while iduronic acid does contain a C5 substituent, its configuration is the opposite of that of glucose. Thus, in the $^{2,5}B$ conformation, the carboxylate is pseudoequatorial, and does not contribute to destabilizing flagpole interactions, making the $^{2,5}B$ conformation as accessible to iduronic acid as it is to xylose (Figure 8c). It is possible that the ability of both xylosides and iduronides to adopt this conformation is the basis for the homology between the xylosidases and iduronidases, and therefore their assignment to the same glycosidase family.

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REFERENCES

1. Freeman, C., and Hopwood, J. J. (1992) *Biochem. J. 282*, 899−908.
2. Shull, R. M., Kakkis, E. D., McEntee, M. F., Kania, S. A., Jonas, A. J., and Neufeld, E. F. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12937−12941.
3. Henrissat, B. (1991) *Biochem. J. 280* (Part 2), 309−316.
4. Henrissat, B., and Bairoch, A. (1993) *Biochem. J. 293* (Part 3), 781−788.
5. Scott, H. S., Ashton, L. J., Evre, H. J., Baker, E., Brooks, D. A., Callen, D. F., Sutherland, G. R., Morris, C. P., and Hopwood, J. J. (1990) *Am. J. Hum. Genet. 47*, 802−807.
6. MacDonald, M. E., Scott, H. S., Whaley, W. L., Pohl, T., Wasmuth, J. J., Lehrach, H., Morris, C. P., Frischauf, A.-M., Hopwood, J. J., and Gusella, J. F. (1991) *Somatic Cell Mol. Genet. 17*, 421−425.
7. Scott, H. S., Anson, D. S., Orsborn, A. M., Nelson, P. V., Clements, P. R., Morris, C. P., and Hopwood, J. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9695−9699.
8. Armand, S., Vieille, C., Gey, C., Heyraud, A., Zeidus, J. G., and Henrissat, B. (1996) *Eur. J. Biochem. 236*, 701−713.
9. Knapp, S., Vocadlo, D., Gao, Z., Kirk, B., Lou, J., and Withers, S. G. (1996) *J. Am. Chem. Soc. 118*, 6804−6805.
10. Unger, E. G., Durrant, J., Anson, D. S., and Hopwood, J. J. (1994) *Biochem. J. 304*, 43−49.
11. Wong, A. W., He, S., and Withers, S. G. (2001) *Can. J. Chem. 79*, 510−518.
12. Wong, A. W., He, S., Brubb, J. H., Sly, W. S., and Withers, S. G. (1998) *J. Biol. Chem. 273*, 34057−34062.
13. Clements, P. R., Muller, V., and Hopwood, J. J. (1985) *Eur. J. Biochem. 152*, 29−34.
14. Whitfield, D. M., Birnbaum, G. I., Pang, H., Baptista, J., and Sarkar, B. (1991) *J. Carbohydr. Chem. 10*, 329−348.
15. Durand, P., Lehn, P., Callebaut, I., Fabrega, S., Henrissat, B., and Mornon, J.-P. (1997) *Glycobiology 7*, 277−284.
16. Brooks, D. A., Fabrega, S., Hein, L. K., Parkinson, E. J., Durand, P., Yogalingam, G., Matte, U., Giugliani, R., Dasvarma, A., Eslahpazire, J., Henrissat, B., Mornon, J.-P., Hopwood, J. J., and Lehn, P. (2001) *Glycobiology 11*, 741−750.
17. Sidhu, G., Withers, S. G., Nguyen, N. T., McIntosh, L. P., Ziser, L., and Brayer, G. D. (1999) *Biochemistry 38*, 5346−5354.
18. Sabini, E., Sulzenbacher, G., Dauter, M., Dauter, Z., Jorgensen, P. L., Schulein, M., Dupont, C., Davies, G. J., and Wilson, K. S. (1999) *Chem. Biol. 6*, 483−492.

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