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Glycoside Cleavage by a New Mechanism in Unsaturated Glucuronyl Hydrolases

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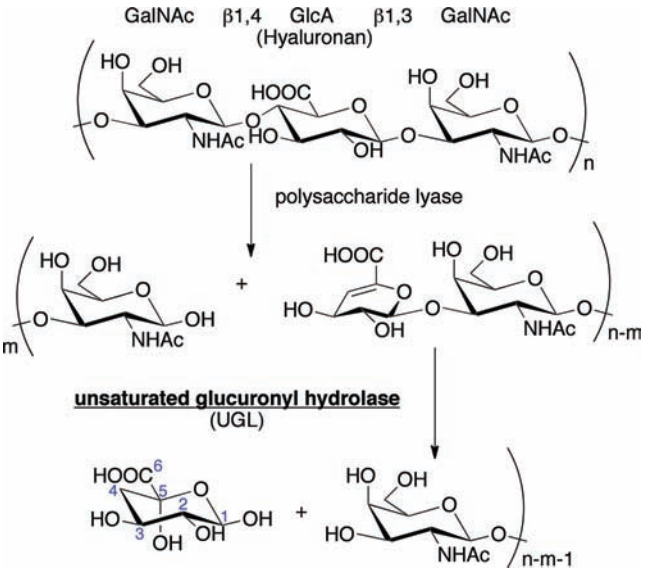
Supporting Information

ABSTRACT: Unsaturated glucuronyl hydrolases (UGLs) from GH family 88 of the CAZy classification system cleave a terminal unsaturated sugar from the oligosaccharide products released by extracellular bacterial polysaccharide lyases. This pathway, which is involved in extracellular bacterial infection, has no equivalent in mammals. A novel mechanism for UGL has previously been proposed in which the enzyme catalyzes hydration of a vinyl ether group in the substrate, with subsequent rearrangements resulting in glycosidic bond cleavage. However, clear evidence for this mechanism has been lacking. In this study, analysis of the products of UGL-catalyzed reactions in water, deuterium oxide, and dilute methanol in water, in conjunction with the demonstration that UGL rapidly cleaves thioglycosides and glycosides of inverted anomeric configuration (substrates that are resistant to hydrolysis by classical glycosidases), provides strong support for this new mechanism. A hydration-initiated process is further supported by the observed UGL-catalyzed hydration of a C-glycoside substrate analogue. Finally, the observation of a small β-secondary kinetic isotope effect suggests a transition state with oxocarbenium ion character, in which the hydrogen at carbon 4 adopts an axial geometry. Taken together, these observations validate the novel vinyl ether hydration mechanism and are inconsistent with either inverting or retaining direct hydrolase mechanisms at carbon 1.

Glycosaminoglycans (GAGs), composed of alternating uronic acids and amino sugars, are present in the extracellular matrix of all mammalian tissues.[1] During extracellular bacterial infection GAGs represent a barrier to bacterial motility, so GAG-degrading enzymes are virulence factors.[2,3] Additionally, GAGs represent an abundant potential energy source. Bacteria thus have a dual incentive to degrade these polysaccharides, so inhibitors of the enzymes involved have potential as bacteriostatic agents. The bacterial pathway for the degradation of GAGs has no mammalian homologue, and thus presents an appealing target for drug development. This pathway involves two enzymes (Scheme 1).[4] The first of these is a polysaccharide lyase, which has been shown to act via an E1cb mechanism and generates an unsaturated sugar at the nonreducing terminus.[5] This class of enzyme is usually specific to a particular GAG.[6] The second enzyme, unsaturated glucuronyl hydrolase (UGL) in GH family 88 of the Carbohydrate Active Enzymes Database (http://www.cazy.org/),[7] is often less stringent in its substrate specificity[8] and thus can turn over the products of many lyases; it has been proposed to catalyze

Scheme 1. Enzymatic Pathway for Degradation of a Representative Glycosaminoglycan by Bacteria, Showing the Role of UGL and the Carbon Numbering in the Final Product (in Blue)



hydration of this terminal unsaturated sugar to effect cleavage of those partially degraded disaccharides.[9] However, limited direct evidence exists for this mechanism, which is the focus of the present work. If the novel mechanism is confirmed then this pathway effects degradation of a complex mix of polysaccharides to individual monomers without the use of any classical glycoside hydrolases.

The proposed mechanism[9] (Scheme 2) begins with protonation at carbon 4 of the double bond between carbons 4 and 5 of the sugar at the nonreducing terminus. This generates an oxocarbenium ion-like intermediate across carbon 5 and the endocyclic oxygen, which is subsequently attacked at carbon 5 by water with general base catalysis. The resulting product is a hemiketal that can spontaneously undergo ring opening, leading to cleavage of the glycosidic bond through an intermediate hemiacetal. The free unsaturated sugar product can then be further hydrated in a spontaneous process, leading to several cyclic products in equilibrium, as seen in product ^{1}H NMR spectra.

Previous evidence[9] for this mechanism is somewhat inconclusive. The mechanism was proposed on the basis of the arrangement of side chains around the bound substrate in the

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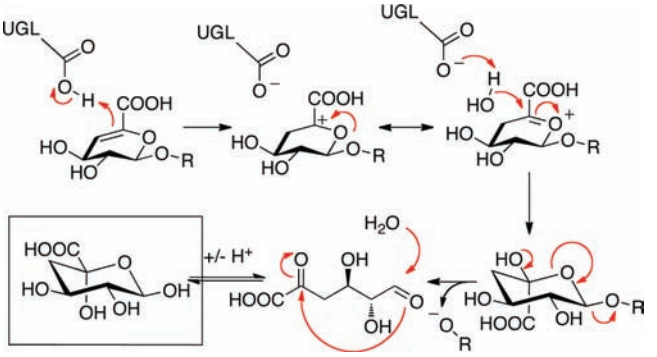
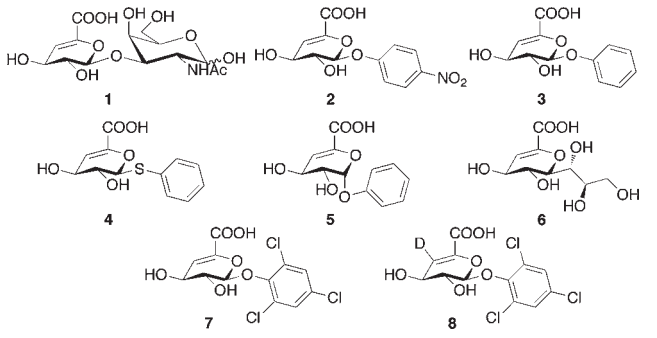
Scheme 2. Proposed Mechanism for UGL[a]



[a] See ref 9. The final product can have any combination of stereochemistries at positions 1 and 5; only the dominant product is shown here. Collapse of the hemiketal intermediate (lower right) is shown as a single concerted step for simplicity.

Scheme 3



crystal structure of a mutant UGL complex. It was noted that, in this structure, no potential acid/base or nucleophile residues are situated close to the anomeric center. The evidence consisted of the demonstration of incorporation of ^{18}O at carbon 5 when the reaction was carried out in isotopically labeled water, as deduced from fragmentation patterns in product mass spectra. However, the possibility that this incorporation occurred by exchange into the product after the enzymatic reaction was not considered, leaving some doubts. Nonetheless, the mechanism proposed is ingenious and intriguing and merits more detailed mechanistic analysis.

To establish this mechanism more rigorously, UGL from *Clostridium perfringens* was heterologously expressed in *Escherichia coli,* purified as an N-terminal His_6-tagged protein, and confirmed as active with a natural substrate, **1** (Scheme 3). A synthetic substrate, 4-nitrophenyl-4-deoxy-α-L-*threo*-hex-4-enopyranosiduronic acid (**2**), was prepared as described in the Supporting Information (SI) and confirmed to be cleaved by the enzyme (Table 1). This and related substrates were subsequently used to probe the sites of nucleophilic attack and proton addition during the enzyme-catalyzed hydrolysis reaction.

Enzymatic hydrolysis of the synthetic substrate **2** was carried out in deuterated water to test the hypothesis that the proton at carbon 4 is added directly by the enzyme rather than in a late-stage spontaneous process (keto−enol tautomerization in free solution), as would be expected if a conventional mechanism holds. The ^1H NMR spectrum of the resultant product (Figure 1, bottom spectrum) showed the complete absence of the peak

Table 1. Steady-State Kinetic Parameters for *C. perfringens* UGL with the Substrates Shown in Scheme 3

substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}\ s^{-1}$)
1	8.5 ± 0.3	3.0 ± 0.2	2.8 ± 0.3
2	2.05 ± 0.06	0.26 ± 0.02	7.9 ± 0.8
3	4.3 ± 0.2	3.2 ± 0.4	1.3 ± 0.2
4	9.3 ± 0.2	4.8 ± 0.3	1.9 ± 0.2
5	0.6 ± 0.1	38 ± 9	0.015 ± 0.006
6	n.d.[a]	n.d.	0.0133 ± 0.0002
7	0.31 ± 0.02	0.57 ± 0.04	0.54 ± 0.07
8	n.d.	n.d.	0.57 ± 0.09[b]

[a] n.d. = individual values not determined: kcat/Km derived from initial slope. [b] Calculated from k_{cat}/K_M for substrate 7 and the measured kinetic isotope effect.

assigned to the axial proton at carbon 4 in a control hydrolysis reaction (Figure 1, center spectrum), while the peak assigned to the equatorial proton at the same position was unchanged in intensity but had lost a geminal coupling partner (red dashed box). The signal assigned to the proton at carbon 3 also lacked the trans-diaxial coupling seen in its protio equivalent. This result confirms the stereospecific incorporation of this deuterium, indicative of an enzymatic addition. The axial placement of this deuterium matches the location of the proposed catalytic acid/base residue at the *re* face of carbon 4 in the crystal structure, lending support to both the mechanism and the identification of this residue as the catalytic acid.

The site of nucleophilic attack of the water cannot be determined reliably because of the subsequent exchange processes noted earlier. However, a more stable enzymatic product was obtained by addition of 10% (v/v) methanol to reaction mixtures containing synthetic substrate (**2** or **3**) and UGL. This methanol can act as an alternate nucleophile in competition with water, giving rise in this case to a mixture of the methyl ketal analogue of the hemiketal initial product (Scheme 2, lower right) and the regular hydrolysis product. Attempts to purify either such product (from **2** or **3**) were unsuccessful, as these species were stable for only a few hours in solution, with degradation observable after they were left overnight at 4 °C. ^1H NMR analysis was therefore carried out on the crude product mixture containing predominantly hydrolysis products with only a small proportion of the semistable analogue, as well as traces of unreacted starting material. A new set of peaks was observed (Figure 1, top spectrum) that was not present in the hydrolysis control, with most of these minor new peaks closely resembling those of the final hydrate product and not the starting material. An additional 3H singlet from the *O*-methyl moiety was also observed.

This new set of peaks showed the expected set of correlation spectroscopy (COSY) cross-peaks for the product of methanol addition at carbon 5. The OMe singlet peak showed a nuclear Overhauser spectroscopy (NOESY) cross-peak to the axial proton at carbon 4, consistent with an equatorial geometry following addition to the *si* face of the double bond at carbon 5. This is the product of attack predicted on the basis of the location of a water molecule in the crystal structure and represents an overall syn addition of H_2O in the natural reaction. If a classical glycosidase mechanism were operative, addition of the methyl group would occur at carbon 1. Standards for addition of methanol to carbon 1 were thus synthesized with both stereochemistries, and the resultant ^1H NMR spectra allowed these to be ruled out as the product of the enzymatic reaction. A standard for addition of methanol to carbon 5

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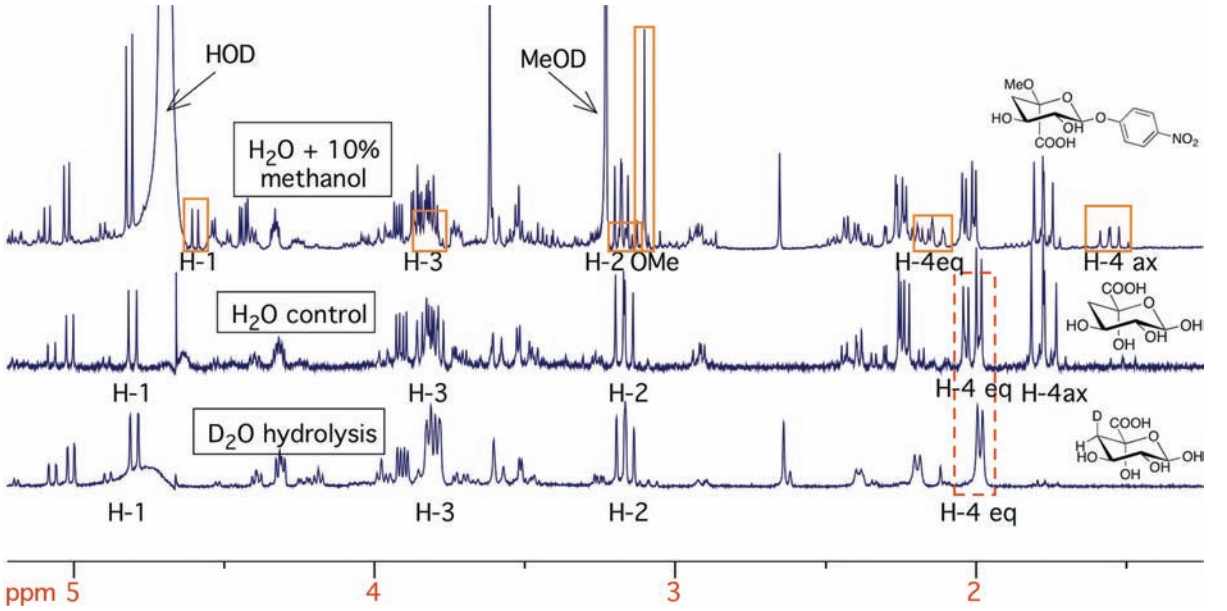

Figure 1. Overlay of ^{1}H NMR spectra recorded in D_2O for UGL-catalyzed degradation of **2** in 10% (v/v) methanol/H_2O (top), H_2O (center), and D_2O (bottom). Assignments of main peaks are shown below each spectrum; for the 10% methanol product, the identified peaks are those of the methanol-adduct minor product and are boxed in orange. The dashed red box indicates the main signal of interest in the D_2O reaction, that of the remaining proton on carbon 4.

was also synthesized by acid-catalyzed addition of methanol to the peracetylated methyl ester of **3**. The product formed under these conditions had the opposite stereochemistry at carbon 5, with the axial location of the methyl group shown by a NOESY cross-peak to the equatorial proton at carbon 4 as well as the axial protons at carbons 3 and 1. This isomer was stable in solution, showing no signs of degradation after several months. The formation of the unstable stereoisomer in the enzymatic experiments thus provides additional evidence that this (equatorial) product is formed by the enzyme and not in free solution.

The observation of this methanol syn-addition product also excludes the possibility of a mechanism involving a covalent glycosyl−enzyme intermediate formed by syn addition of the general acid catalyst residue across the double bond, as has previously been observed for hydration of vinyl ethers in 1,2-glycals by retaining glycoside hydrolases.[10,11] If such an intermediate had been formed and carbon 5 of the sugar had been attacked by methanol, the stable anti-addition product would have been seen. If this same intermediate had been formed and then attacked by the methanol at the acyl carbon of the side chain, a methyl ester would have formed, and the enzyme would have been unable to catalyze the multiple turnovers observed (this situation also would not account for the incorporation of methanol into the product).

As a further probe of the novel mechanism, several compounds were synthesized and tested, each of which was predicted to be accepted as a substrate by UGL *only* if this novel mechanism were acting. All of these were indeed seen to be turned over (Table 1). The first of these was thiophenyl glycoside **4**. Thioglycosides are poor substrates for conventional glycoside hydrolases and are frequently used as inert substrate analogues for crystallographic studies. These are, however, cleaved by enzymes using eliminative mechanisms such as those in the GH4 family in the CAZY database[12] and polysaccharide lyases.[13] Indeed, **4** was an excellent substrate and was turned over by UGL with a k_{cat}/K_M value superior to that of its oxygen analogue **3**.

Compound **5**, the alternate anomer of phenyl glycoside **3** having stereochemistry opposite to that of the natural substrate at carbon 1, was also accepted by UGL as a substrate, albeit with a very high apparent K_M. Fitting of the available data suggested a k_{cat} in the same approximate range as observed for other substrates tested. Turnover of this substrate would be highly unlikely in a glycoside hydrolase that acts directly at the anomeric center, as the arrangement of catalytic residues would have to be optimized for both orientations. However, this is the expected result for the proposed mechanism, as the hydration reaction occurs at a site remote from the inverted stereocenter. The high K_M is also expected, for while the catalytic mechanism is not reliant on the correct stereochemistry at this center, the binding is. Such hydrolysis of both anomers of an activated substrate has been seen before for other glycoside-cleaving enzymes that act through a mechanism other than direct hydrolysis at the anomeric carbon, such as the GH4 family in the CAZY database.[14,15]

Finally, commercially available 2,6-anhydro-3-deoxy-D-glycero-D-galacto-non-2-enoic acid (Kdn2en, **6**), which resembles the unsaturated sugar moiety of the natural substrate but has a C−C-linked glycerol chain in place of a leaving group, was hydrated by the enzyme but could not undergo the subsequent rearrangements seen for the other substrates. Formation of the hydrated product was confirmed by mass spectrometry and ^{1}H NMR analysis. The individual kinetic parameters for this hydration could not be determined because of its poor binding. However the k_{cat}/K_M value was similar to those seen for other substrates (the rate increased linearly with substrate concentration up to 7.5 mM).

Having established the fundamental mechanism, we sought further insights into the rate-determining step and transition state using kinetic isotope effect (KIE) analysis. The solvent KIE was previously measured for *Bacillus* sp. GL1 UGL to be 2.1 for k_{cat} and 2.2 for k_{cat}/K_M,[9] but this type of experiment is unable to distinguish between the two mechanisms, both of which involve proton addition in the rate-determining step. To further this work,

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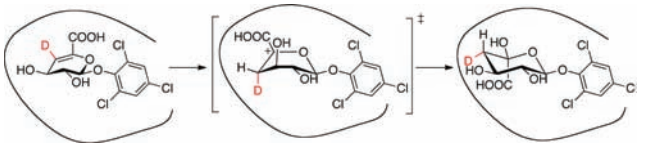
Scheme 4. Deduced Conformation of the Transition State Leading to the Observed KIE (the Enzyme Active Site Is Represented by an Incomplete Border)



the pair of substrates **7** and **8**, having either hydrogen or deuterium at carbon **4**, were synthesized as described in the SI. The trichlorophenyl leaving group was used here to allow ease of synthetic access to the product through hydrogenation. The KIE on k_{cat}/K_M was measured spectrophotometrically in individual reactions to be 1.06 ± 0.02 and confirmed by direct competition in one reaction monitored by ^{1}H NMR analysis (1.08 ± 0.03). This small effect was initially surprising, as an inverse α-secondary KIE had been anticipated. However, previous measurements of KIEs for hydration of C−C double bonds under acidic conditions have revealed values around unity.[16,17] This low value is attributed to equal but opposite effects from a change in hybridization from sp^2 to sp^3 at the site of substitution (giving an inverse α-secondary effect) and hyperconjugation with the adjacent carbocation at carbon 5 (giving a normal β-secondary effect). Since such β-secondary KIEs are highly dependent on geometry because of the need for alignment of the C−D bond and the adjacent empty p orbital, the finding of a net KIE of near unity implies a relatively large β-secondary effect to balance the expected inverse α-secondary effect. In turn, this suggests a transition-state geometry such as that shown in Scheme 4, with the C−D bond axial.

In summary, evidence has been provided here that clearly demonstrates the use of a novel hydration mechanism by unsaturated glucuronyl hydrolases to effect cleavage of glycosidic bonds: the proton addition at carbon 4 has been shown to occur in a stereospecific manner, and nucleophilic attack has been shown to occur from the same face at carbon 5. The reaction directly catalyzed is the syn hydration of a vinyl ether to give an unstable hemiketal, and it is the collapse of this species that leads to glycosidic bond cleavage. The present data do not allow us to determine whether this collapse occurs on the enzyme or free in solution. The formation of a glycosyl−enzyme intermediate through *syn* addition of the general acid catalyst residue was ruled out, in contrast to the established mechanism for enzymatic hydration of glycals. A secondary deuterium kinetic isotope effect on k_{cat}/K_M from a 4-deuterated substrate was observed, also consistent with the proposed mechanism involving an oxocarbenium ion-like transition state, indicating that this deuterium adopts an axial geometry in the transition state. The leaving group was seen to be unimportant in the catalytic mechanism, being important only in binding. In fact, in the case where it was replaced by a carbon-linked glycerol chain, the enzyme could still catalyze the hydration reaction. Confirmation of this mechanism therefore further expands the body of known enzymatic strategies employed for cleavage of glycosides.

■ ASSOCIATED CONTENT

Supporting Information. Overlay of ^{1}H NMR spectra for the 10% methanol product with all controls and standards; COSY and NOESY spectra; details of cloning, enzyme purification, compound synthesis, and characterization; and kinetics and enzymatic reaction conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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