Mechanistic Investigations of Unsaturated Glucuronyl Hydrolase from Clostridium perfringens*5

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Experiments were carried out to probe the details of the hydration-initiated hydrolysis catalyzed by the Clostridium perfringens unsaturated glucuronyl hydrolase of glycoside hydrolase family 88 in the CAZy classification system. Direct 1H NMR monitoring of the enzymatic reaction detected no accumulated reaction intermediates in solution, suggesting that rearrangement of the initial hydration product occurs on-enzyme. An attempt at mechanism-based trapping of on-enzyme intermediates using a 1,1-difluoro-substrate was unsuccessful because the probe was too deactivated to be turned over by the enzyme. Kinetic isotope effects arising from deuterium-for-hydrogen substitution at carbons 1 and 4 provide evidence for separate first-irreversible and overall rate-determining steps in the hydration reaction, with two potential mechanisms proposed to explain these results. Based on the positioning of catalytic residues in the enzyme active site, the lack of efficient turnover of a 2-deoxy-2-fluoro-substrate, and several unsuccessful attempts at confirmation of a simpler mechanism involving a covalent glycosyl-enzyme intermediate, the most plausible mechanism is one involving an intermediate bearing an epoxide on carbons 1 and 2.

One important role of polysaccharides is to provide structural support for cells. For mammalian tissue, the most important class of structural polysaccharides is that of the glycosaminoglycans (1, 2). The dense matrix of these glycosaminoglycans forms an important extracellular matrix for cells. For mammalian tissue, the most important class of structural polysaccharides is that of the glycosaminoglycans (1, 2). The dense matrix of these glycosaminoglycans also provides a physical barrier to the spread of extracellular bacterial infection. To counter this effect, some extracellular bacteria have evolved a metabolic pathway for degrading these glycosaminoglycans, thereby liberating the constitutive monomers for use as an energy source.

This bacterial pathway for degradation of glycosaminoglycans includes two key steps (3) as follows: depolymerization by a lyase using an E1cB mechanism and then hydration-initiated cleavage of the resultant unsaturated terminal monomer. The enzymes responsible are mechanistically very different from the endogenous mammalian enzymes responsible for glycosaminoglycan remodeling. This mechanism resembles the fact that these enzymes are virulence factors (4, 5) make this pathway an attractive target for small molecule inhibitors that could prevent or slow bacterial growth. This approach is particularly applicable to the prevention of infection by opportunistic pathogens following surgery.

Design of such inhibitors relies on a thorough understanding of the underlying chemical mechanism of the enzyme. The mechanisms of the enzymes that carry out the first step in the bacterial pathways for degradation of various glycosaminoglycans, the polysaccharide lyases, are well understood and have been covered in several reviews (6–9). However, the mechanisms of the enzymes carrying out the second step in these pathways, the unsaturated glucuronyl hydrolases (UGL),2 remain enigmatic.

On the basis of catalytic residue placement in a substrate-bound crystal structure, a hydration-based mechanism was proposed (10) and appears to be currently accepted for UGL. This mechanism, shown in Fig. 1, is initiated by protonation of the double bond between carbons 4 and 5 in the unsaturated glucuronide (ΔGlcUA) substrate. This is followed by quenching of the oxocarbenium ion by attack of water at carbon 5, resulting in net hydration of the double bond. In this mechanism, the reaction may pass through an oxocarbenium ion-like transition state for a concerted addition or a short lived high energy intermediate, with transition states for its formation and breakdown having a large degree of oxocarbenium ion-like character. The product of this hydration is an unstable hemiketal glycoside, which rearranges to a keto-hemiacetal by opening of the pyranose ring. Further rearrangement of the hemiacetal results in cleavage of the glycosidic bond. The resultant linear keto-aldehyde then hydrates and re-cyclizes in aqueous solution (11).

* The abbreviations used are: UGL, unsaturated glucuronyl hydrolase; KIE, kinetic isotope effect; Kdn, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid; AroA, enolpyruvylshikimate-3-phosphate synthase; ΔGlcUA, for 4S-unsaturated gluconic acid (4-deoxy-α-L-threono-hex-4-enopyranosiduronic acid).

** Significance: Understanding of the mechanism of UGL may allow design of bacteriostatic agents.
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The crystal structure with substrate bound showed two aspartate residues to be located in the UGL active site, both of which were previously shown by mutagenesis to be catalytically important (12). However, the proposed mechanism provides a convincing role for only one of these aspartates.

The initial hydration reaction was subsequently confirmed experimentally (11) and shown to take place in an overall syn fashion. Experimental data published to date are consistent with a positively charged transition state, as evidenced by a linear free-energy relationship with a shallow positive slope with a positively charged transition state, as evidenced by a linear free-energy relationship with a shallow positive slope (13).

An attempt at design of inhibitors based on this mechanism and its implied transition states was unsuccessful (13), suggesting that this mechanism is incomplete. If the mechanism shown in Fig. 1 is not correct, then the following question remains: How does UGL carry out its hydration reaction? Tied to this is a further question regarding the exact role of the second catalytically important aspartate residue in the UGL active site, which was suggested to somehow stabilize the transition state for hydration. The manner in which this stabilization might occur is not intuitive. The studies presented here will attempt to address both of these questions.

EXPERIMENTAL PROCEDURES

Source of Compounds—Thiophenyl-ΔGlcUA, 4-nitrophenyl ΔGlcUA, 3-nitrophenyl ΔGlcUA, 2,4,6-trichlorophenyl ΔGlcUA, and 2,4,6-trichlorophenyl 4-2H-ΔGlcUA were synthesized as described previously (11, 13). 2,3-Difluoro-Kdn was synthesized as described previously (11, 13). 2,3-Difluoro-Kdn was synthesized as described previously (11, 13). The crystal structure with substrate bound showed two aspartate residues to be located in the UGL active site, both of which were previously shown by mutagenesis to be catalytically important (12). However, the proposed mechanism provides a convincing role for only one of these aspartates.

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General Kinetic Methods—Expression and purification of Clostridium perfringens UGL was carried out as reported previously (11). Kinetic measurements were performed in optically matched 1-cm path length reduced volume quartz cuvettes using a Varian Cary 4000 spectrophotometer with automatic cell changer and Peltier temperature controller at 37 °C in 50 mM MES/NaOH buffer, pH 6.6, with 1 mg/ml bovine serum albumin (BSA) and 1 μM UGL unless otherwise specified. Reaction mixtures were allowed to preincubate for 5 min before adding enzyme to start the reaction. In those cases where reaction time courses were linear over a sufficiently long time, several rates were measured simultaneously using an automated cell changer. All nonlinear regression was performed using GraFit 5.0 (Erithacus Software Ltd.).

Inhibition—The K_i value was initially estimated using substrate at slightly above K_m values and a serial dilution of inhibitor, with an assumption of competitive inhibition. Following this, inhibition was assayed with a matrix of substrate and inhibitor concentrations bracketing K_m and the estimated K_i values. These rates were fit to modified Michaelis-Menten equations describing reaction in the presence of competitive, noncompetitive, and mixed type inhibition using nonlinear regression. The equation giving the lowest errors was deemed to be the most appropriate, the selection of which was corroborated by plotting 1/rate against inhibitor concentration (a Dixon plot) and observing the intersection of plots at X = −1/K_i and Y = 1/V_max.

Inactivation—Compounds designed as potential mechanism-based inactivators of UGL were tested for their ability to induce time-dependent loss of enzyme activity. Samples at varied inhibitor concentrations were incubated at 30 °C with enzyme (4 μM), using the buffer described for standard Michaelis-Menten kinetics, and aliquots were removed at time intervals to test for residual enzyme activity. For each time interval, 20 μl of the inactivation reaction mixture was added to 180 μl of a pre-prepared substrate/buffer mix with substrate in a large excess over its K_m value (4-nitrophenyl ΔGlcUA, 1.875 mM), and absorbance change was monitored for 1–2 min at 37 °C.

Data for each inactivator concentration were fit to an equation describing first order decay with offset. As no clear inactivation at varied concentrations was observed, these first order rates were not further processed. For all reactions where inactivation was observed, the pH was tested using a pH-fix strip (Macherey-Nagel, Germany) before and after incubation. For inactivator concentrations close to or above the buffer concentration, the inactivator solution was adjusted to pH 6.5 by mixing equimolar solutions of the inactivator as a free acid and as its sodium salt.

UGL Monitoring by 1H NMR—UGL was exchanged into 50 mM phosphate buffer, pH 7.1, with 1 mM β-mercaptoethanol in 100% D_2O by repeated spin filtration using a 30-kDa cutoff spin filter at 4 °C, and then enzyme concentration was determined by A_280 and BSA added from stock (1% w/v, also in D_2O) to a final concentration of 0.1% w/v. All locking, tuning, and shimming of the spectrometer was performed on buffer/BSA mix before addition of the enzyme/buffer/BSA mix to the substrate. Hydrolysis of 3-nitrophenyl ΔGlcUA (4.1 mg) and thiophenyl ΔGlcUA (3.9 mg) by UGL (48.5 μM for 3-nitrophenyl ΔGlcUA and 14 μM for thiophenyl ΔGlcUA) was monitored using 1H NMR with a water suppression pulse program after 0, 5, 15, 30, and 60 min (0, 5, 10, 15, 45, 110, 170, and 266 min for thiophenyl ΔGlcUA, with no water suppression) at 25 °C. The sample was then left at ambient temperature over 66 h (~72 h for thiophenyl ΔGlcUA) before a final spectrum was recorded, to allow time for any slow equilibration. Each spectrum was recorded for 32 scans at 400 MHz in a Bruker Avance 400 with inverse probe. A separate spectrum of each substrate was also recorded in the same buffer conditions without enzyme.

Kinetic Isotope Effects—Stocks of UGL in D_2O (see above) and H_2O were made to the same concentration, as determined by A_280. For solvent KIEs, stock solutions of 4-nitrophenyl ΔGlcUA were prepared in D_2O and H_2O. Reactions in H_2O were measured in buffer at pH 6.6, and those in D_2O were measured in buffer at pH 7.1 (16), using phosphate buffers (direct pH meter readings). For isotope effects on k_cat, initial rates were measured using 2 mM substrate, for which a linear fit was determined in a total of eight replicates. Averaging of these linear rates for each solvent allowed calculation of a ratio and its
standard error. For isotope effects on $k_{cat}/K_m$, the first order rate constant was measured by substrate depletion using 50 $\mu$M substrate. Reactions were monitored using a cell changer containing four reactions at once, with a final total of eight replicates for each solvent. These first order rate constants were also averaged for each substrate, allowing calculation of a ratio and its standard error.

To measure the KIEs from deuterium-for-hydrogen substitution at carbon 1 and carbon 4, stocks of 2,4,6-trichlorophenyl-1,2-2H- and 4-2H-ΔGLcUA, as well as the nondeuterated isotope, were prepared at the same concentrations in H2O. Rates were determined and unsubstituted substrates using a single stock for each solvent. Measurements were alternated between isotopically substituted substrates and enzyme stock to determine the isotope effects on $k_{cat}$, and $k_{cat}/K_m$, respectively. Measurements were alternated between isotopically substituted and unsubstituted substrates using a single stock for each substrate and the enzyme in all reactions to minimize error. Stock solutions were preincubated at 37 °C between assays and mixed before withdrawing solution. To minimize pipetting errors, each stock was made to a concentration that allowed for mixing of large volumes relative to the total, typically 100 $\mu$l each of substrate and enzyme stock for a 200-$\mu$l reaction. Ratios of $k_{cat}$ to $k_{w}$ were determined as detailed for the solvent KIEs (using 50 $\mu$M substrate for the effects on $k_{cat}/K_m$ and 5 mM rather than 2 mM substrate used for the effects on $k_{cat}$, to account for the higher $K_m$ of 2,4,6-trichlorophenyl (ΔGLcUA).

D113G Mutagenesis—The C. perfringens UGL D113G mutant was generated using the QuickChange method. The following primers were used: forward, G GAA AAA GAT ATA GAA TTA GAT CAT CAT GGT TTA GGA TTC TTT TAT TCA GTC ATA ATA TGA AAT ACG TAA TGA ATA CAA GAA TTC TAA ACC ATG ATG ATC TTC TAA TAT ATC TTT TTC C (mismatch in bold). These primers were used for PCR (17 cycles: 30 s, 98 °C denaturation, 1-min gradient 55--65 °C annealing, 7 min 68 °C extension) with the pET28a:UGL plasmid (11) as template (from a 1-ml overnight culture) purified by Qiagen mini-prep kit eluted with 50 $\mu$l of H2O, by Pfu polymerase (50-$\mu$M reaction, 5 $\mu$l of 10× Pfu buffer, 2 $\mu$l of template, 1 $\mu$l each of 10 mM primers, 1 $\mu$l of Pfu enzyme, 4 $\mu$l of 2.5 mM dNTPs). Following confirmation of the PCR product by agarose gel, the PCR product was purified (Qiagen PCR purification kit), and the template was digested using DpnI (40-$\mu$M reaction, 4 $\mu$l of 10× Fast digest buffer, 5 $\mu$l of purified DNA, and 1 $\mu$l of DpnI Fast digest enzyme (Fermentas), reacted overnight at 37 °C). The resulting mutant plasmid was transformed into TOP10 E. coli cells by heat shock (45 °C for 1 min), and the cells were allowed to recover (1 ml of LB media added and then incubated at 37 °C for 1 h with shaking at 225 rpm) before plating on kanamycin (50 $\mu$M) LB selection plates. Colonies were picked and used to inoculate overnight cultures (1 ml of Kan-LB), and plasmids were purified by Qiagen mini-prep kit (eluted with 50 $\mu$l of H2O). Product sequence was confirmed by commercial sequencing with T7 and T7 terminal primers (NAPS unit, University of British Columbia, Canada).

The isolated UGL D113G mutant plasmid was subsequently transformed by electroporation (400 ohms, 25 microfarads, and 2.5 kV) into BL21(DE3) of E. coli cells for expression. The mutant UGL protein was expressed and purified in the same manner as the wild type (11), using a new column to avoid trace contamination with wild-type enzyme. The purification yielded the mutant enzyme was similar to that of the wild type (~5 mg per liter of culture).

Attempted Rescue of Mutant—To test for rescue of catalytic activity, UGL D113G (57 $\mu$m) was incubated at ambient temperature in phosphate buffer (50 mM, pH 5.0) with BSA (0.1% w/v), 4-nitrophenyl ΔGLcUA (10 mM), and a range of potential alternative nucleophiles. Nucleophiles tested were sodium formate (pH 5.0, 1 mM), sodium acetate (pH 5.0, 0.5 mM), methanol (4% v/v), p-mercaptoethanol (1 mM), sodium azide (1 mM), sodium cyanate (0.25 mM), sodium thiocyanate (1 mM), and potassium cyanide (1 mM). Reactions were monitored daily over a week using TLC (3:2:1 butanol/acetic acid/water). Testing in a spectrophotometer was carried out by monitoring release of 4-nitrophenolate at 405 nm by UGL-catalyzed hydrolysis of 4-nitrophenyl ΔGLcUA in the presence of sodium azide and sodium formate buffer, pH 6.6, each at a concentration of 100 mM, in otherwise standard assay conditions. The enzyme was preincubated in the presence of azide or formate for 5 min to allow equilibration before starting the reaction by addition of substrate.

RESULTS AND DISCUSSION

1H NMR Monitoring—When the hydration-initiated mechanism of UGL (10) shown in Fig. 1 was proposed, it was assumed that rearrangement of the initial hydrate product occurs within the enzyme active site. However, given that this hydrate is a hemiketal, it is entirely plausible that this rearrangement could take place free in solution. To test this assumption, the reaction of high concentrations of C. perfringens UGL with 3-nitrophenyl ΔGLcUA and thiophenyl ΔGLcUA, both substrates with high $k_{cat}$ values (11, 13), were directly monitored by 1H NMR in 100% D2O, aiming to observe any free hydrated intermediates. Key spectra are shown in Fig. 2.

Under the conditions employed, 3-nitrophenyl ΔGLcUA was consumed within 5 min and thiophenyl ΔGLcUA within 15 min. Formation of the final product was observed from $t = 0$ in both cases, with no sign of additional peaks below 6 ppm, the spectral region where any sugar intermediate or side product peaks would be expected. In the reaction with thiophenyl ΔGLcUA, peaks arising from side products were observed at around 6.6 and 7.2 ppm after around 10 min, when a large proportion of starting material had already been converted to products. Reaction of 3-nitrophenyl ΔGLcUA did not give rise to these peaks, suggesting that they likely arise from reaction of free thiophenol with buffer components. Indeed a freshly prepared sample of thiophenol in the same buffer showed a small peak in this same region. The peaks between 2.5 and 2 ppm, arising from the hydrogen at carbon 4, were seen to slowly decrease in intensity. This suggests that a small amount of the ketone form of the product is present in solution, despite not being observable directly by NMR, increasing the acidity of this proton and allowing its slow exchange with bulk solvent. By contrast, the lack of proton exchange observed at carbon 2 during this experiment suggests that, in the open chain form of the product, a much larger proportion of the aldehyde at carbon 1 exists as a hydrate compared with the ketone at carbon 5.
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If rearrangement of the hydrated intermediate were to occur free in solution, the half-life would likely be on the same order as that for mutarotation of sialic acid derivatives, which contain an analogous 3-deoxy-2-keto group adjacent to a carboxylic acid at carbon 1. Because the half-life for mutarotation of Neu5Ac at pH 6.7 is around 25 min (17), and that of Kdn appears to be similar (18), it would seem likely that any hydrated intermediate released into solution by UGL would accumulate under the experimental conditions used, and thus become detectable by $^1$HNMR, before formation of the final product. These results were interpreted as suggesting that rearrangement of the hydrated intermediate is indeed catalyzed within the UGL active site, as assumed previously.

**Anomeric Difluoro Substrate**—As a further test for the rearrangement of a hydrated intermediate within the UGL active site, 1-fluoro-$\Delta$GlcUA fluoride was synthesized and tested as a potential mechanism-based trapping agent. As shown in Fig. 3, if this compound underwent UGL-catalyzed hydration and subsequent rearrangements, it would form an activated acyl fluoride in the enzyme active site, which would most likely non-site-specifically inactivate the enzyme by modifying the first encountered active site nucleophile. If, however, rearrangement occurred outside the enzyme active site, then the acyl fluoride would be rapidly quenched by water, thereby preventing inactivation of the enzyme.

Unfortunately, no change in $A_{240}$ (the absorption maximum for the $\Delta$GlcUA carbon 4 to carbon 5 double bond) was observed for 5 mM 1-fluoro-$\Delta$GlcUA fluoride incubated with 80 $\mu$M UGL for 95 min under otherwise standard assay conditions, showing that this compound is not efficiently turned over as a substrate by UGL. This is unlikely to be due to poor binding because both anomers of $\Delta$GlcUA fluoride have previously been shown to bind to the UGL active site (13). The disappointing lack of turnover was attributed to the substantial destabilization of the positively charged transition state afforded by the two fluorine atoms, consistent with observations of slow turnover of 2-fluorosugars (see below) and glycosyl fluorides by UGL (13). Importantly, no sign of inactivation above background levels was observed, even at concentrations of 20 mM over a total time of 2 h, as shown in Fig. 4. Above this concentration, however, rapid inactivation of UGL was observed, at a rate that did not appear to be concentration-dependent. Inactivation is therefore likely not enzyme mechanism-based. It was also not due to acidification of the reaction medium, as no change in the pH of the reaction mixture was observed over the course of the inactivation. The mechanism of nonspecific inactivation remains unclear, but it may arise from a physical effect such as substrate analog-promoted aggregation or adhesion to the tube surface at these high concentrations. An analogous explanation was previously offered to explain the apparent inactivation of a yeast $\alpha$-glucosidase by a similar 1-fluoro-glycosyl fluoride (19). Furthermore, the inactivation reported in Ref. 19 occurred in a nonreproducible manner, consistent with the variations in inactivation behavior seen here. The effect may also have its origins in a salt effect of some sort. Indeed, addition of NaCl was also shown to inhibit turnover by UGL (data not shown), suggesting that high ionic strength is destabilizing for this enzyme.

**2-Fluoro Substrate**—A further fluorinated sugar tested as a potential substrate for UGL was 2,4-dinitrophenyl 2-$\Delta$GlcUA. Compounds of this class are typically mechanism-based inactivators of retaining-type glycoside hydrolases and poor substrates of inverting-type glycoside hydrolases (20). However, they would be expected to be efficiently degraded by a hydration mechanism such as that proposed for UGL. Surprisingly, although UGL-catalyzed turnover of 2,4-dinitrophenyl 2-$\Delta$GlcUA did occur, it was extremely slow and detectable only when elevated enzyme concentrations were employed and when monitoring the sensitive 2,4-dinitrophenolate chromophore. A $k_{cat}$ value of $0.93 \times 10^{-4}$ s$^{-1}$ (0.81 per day) was measured, some 15,000-fold lower than that of its 2-hydroxy analog (13). By contrast, binding was not significantly different from that of the 2-OH analog ($K_m = 160 \pm 20 \mu$M) (13), as shown by a $K_J$ of $150 \pm 10 \mu$M ($K_J$ being used as a surrogate for $K_{cat}$ which could not be accurately measured directly as rates below $V_{max}$ would have too high an error). Although some rate reduction would be expected as a consequence of fluorine introduction near the site of positive charge development at the transition state, such exceptionally slow turnover of a 2-deoxy-2-fluoro substrate analog is not con-

![FIGURE 1. Currently accepted mechanism for hydrolysis by UGL, initiated by direct hydration of the carbon 4 to carbon 5 double bond.](image)
FIGURE 2. $^1$H NMR monitoring of ΔGlcUA glycoside hydrolysis by a high concentration of UGL. A, 3-nitrophenyl ΔGlcUA; B, thiophenyl ΔGlcUA. A water suppression pulse program was used for automated variable delay recording of spectra for the 1st h, followed by manual recording of later spectra using a standard proton pulse program with updated tuning and shimming. The overall reaction and main products are as shown top left, with main peaks labeled in the top spectrum (products) and lowest spectrum (starting material). The H-1 peak label for the products is adjacent to the relevant peak, which overlaps substantially with the HOD residual solvent peak and so is also suppressed in the first product spectrum.

FIGURE 3. Anticipated reaction scheme for inactivation of UGL by 1-fluoro-ΔGlcUA fluoride. Formation of the highly reactive acyl fluoride moiety in the enzyme active site would lead to nonspecific derivatization of nucleophilic amino acid side chains close to the active site, some of which will inactivate the enzyme.
The measured isotope effects from deuterium-for-hydrogen substitutions at carbon 1 and carbon 4 are clearly different for $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values. These parameters reflect effects on transition state stability for the overall rate-determining step and the first irreversible step, respectively. That the observed effects on these parameters are clearly different means that the mechanism of UGL must have a first irreversible step that is separate from the overall rate-determining step. This conclusion is inconsistent with the directly hydration hypothesis shown in Fig. 1, where the first irreversible step is assumed to also be the overall rate-determining step. Furthermore, none of the steps following hydration in this mechanism appear plausible as an overall rate-determining step.

The first irreversible step of UGL-catalyzed hydration could plausibly be proton donation to the substrate double bond between carbons 4 and 5, as previous investigations of vinyl ether hydration have shown such a protonation to be largely irreversible (25–27). This is consistent with the previously published isotope effect at carbon 4, which was interpreted as a combination of an inverse secondary isotope effect from changes in hybridization at C4 and a normal secondary effect from hyperconjugation (11). The isotope effect from deuterium-for-hydrogen substitution at carbon 1 on such a protonation would be expected to be negligible, as there is no adjacent vacant orbital for hyperconjugation. Note that the product of this proton donation is not necessarily an oxocarbenium ion intermediate, as the reaction may be passing through an oxocarbenium ion-like transition state en route to a more stabilized intermediate (see below for discussion of possible intermediates).

The overall rate-determining step of UGL also appears to involve development of positive charge at carbon 5, but without the associated change in hybridization at carbon 4, as shown by the strong normal β-secondary isotope effect from a deuterium—for-hydrogen substitution at carbon 4. This effect is stronger than expected at the limit of a β-secondary effect (28) but is still consistent with a hyperconjugation effect. The inverse effect from deuterium at carbon 1 on this parameter is more difficult to interpret. The magnitude of the effect is likely too large to arise from inductive destabilization of the positively charged transition state. One possible interpretation is a nucleophilic attack at carbon 1 during this step, with an associative transition state. Potential candidates for this nucleophile are further discussed below.

Importantly, these results are not consistent with the direct hydration mechanism shown in Fig. 1, with an oxocarbenium ion-like species as either the intermediate or transition state. In the hypothetical case of an oxocarbenium ion intermediate, the quenching of this intermediate by water is highly unlikely to be the overall rate-determining step. Indeed, the transition state for the subsequent attack by water would contain less positive charge than does the formal oxocarbenium ion in the intermediate, and so an inverse KIE on $k_{\text{cat}}$ from deuterium for hydrogen substitution at carbon 4 would be expected, which is not the normal effect seen. In the case of a direct hydration with an oxocarbenium ion-like transition state and no formal intermediate, the first irreversible step would be the same as the overall rate-determining step, and so the KIEs on each step would be the same.
Catalysis by the enzyme enolpyruvylshikimate-3-phosphate synthase (AroA) provides an interesting comparison with UGL. Extensive calculations of the reaction coordinate for both the nonenzymatic acid-catalyzed (29) and AroA-catalyzed (30) cases showed a change in the nature of the transition states, with experimental KIE data in good agreement. Both reactions proceed through a slow irreversible initial protonation followed by fast attack of the nucleophile on the carbocation, but the enzymatic case exhibited a much earlier transition state, with the C–H bond order of 0.24 versus 0.6 in the nonenzymatic case. This earlier transition state arose from substantial stabilization of the carbocation intermediate, as well as the transition state leading to it, by an “electrostatic sandwich” of two carboxyl-containing side chains. Note that although UGL also contains two such side chains of catalytic importance in the active site, their arrangement is very different. The KIE observed in AroA for two deuteriums in the equivalent position to carbon 4 in ΔGlcUA was 0.990 in the enzymatic case and 1.04 in the nonenzymatic case. This small value reflects a similar balancing of hyperconjugation and hybridization effects, with the lower contribution from hyperconjugation in the earlier transition state of the enzymatic case perhaps reflecting a more substrate-like than product-like conformation, thereby limiting orbital overlap. In this case, there are also two deuteriums in the substrate that can give an effect from change in hybridization, whereas only one of these can be configured appropriately to yield the orbital overlap necessary for hyperconjugation.

Finally, solvent KIEs have previously been measured for Bacillus sp. GL1 UGL to be around 2.5 for both $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ (10). Similar values were found for C. perfringens (2.51 ± 0.05 and 2.69 ± 0.12, respectively). Because these effects arise from global replacement of all exchangeable hydrogens with deuterium, they are difficult to interpret. Beyond suggesting that proton transfer events are involved in both of the kinetically important steps, few conclusions can be drawn from this. Notably, solvent KIEs for the few cases of hydrolysis of vinyl ether acetals that have been determined to proceed by initial hydrolysis of the acetal moiety are around 0.37 to 0.55 (26, 31, 32), but values for hydrolysis of vinyl ethers range from 1.56 to 7.1 (26, 31–34). The values determined for UGL clearly fall within the latter range rather than the former, and so are more consistent with a hydration mechanism than a more traditional glycoside hydrolase mechanism, which would act at the acetal first.

Taken together, these kinetic isotope effects show that the hydration reaction catalyzed by UGL includes two separate kinetically relevant steps and does not occur by direct hydration. These steps are likely an initial protonation-driven addition to the double bond between C4 and C5 to form a stabilized intermediate, followed by hydrolysis of this intermediate to give the hydrated C5 hemiketal glycoside. Candidates for this stabilized intermediate will be discussed below.

**Proposed Mechanisms**—The results presented here, as well as those in previous publications (11, 13), suggest a mechanism for UGL that is more complicated than the direct hydration mechanism shown in Fig. 1. Most likely this mechanism involves formation of an intermediate to stabilize the positive charge that develops at carbon 5 after protonation at carbon 4 but before nucleophilic attack by water. Given that a second aspartate residue is located in the active site of the Bacillus sp. GL1 UGL crystal structure and has been shown by mutagenesis to be vital for catalysis, it is parsimonious to assign this residue (referred to here as the “unassigned aspartate”) to avoid confusion with numbering from enzymes across different species, Asp-88 in Bacillus sp. GL1 and Asp-113 in C. perfringens) a role in formation of this postulated intermediate within the hydration reaction. Two potential mechanisms are proposed here to account for all kinetic data.

The first such mechanism, shown in Fig. 5A, involves attack of the unassigned aspartate at the anomeric carbon with concomitant opening of the pyranose ring, giving a 5-keto glycosyl-enzyme intermediate. This intermediate can then be hydrated at the ketone and the pyranose ring re-formed to expel the unassigned aspartate from the anomeric carbon and form the same hydrated intermediate as in the direct hydration mechanism of Fig. 1, with subsequent steps being the same.

This mechanism can account for the isotope effects on $k_{\text{cat}}/K_m$ from deuterium-for-hydrogen substitution at carbon 4 through formation of positive charge at carbon 5 following protonation. The very small normal effect on $k_{\text{cat}}/K_m$ from deuterium-for-hydrogen substitution at carbon 1 is consistent with a weakly dissociative, almost concerted, transition state for the nucleophilic attack at carbon 1 by the unassigned aspartate. Furthermore, assuming formation of the endocyclic oxygen to carbon 1 bond is sufficiently early on the reaction coordinate for the second step, this mechanism can also provide an explanation for the KIEs on $k_{\text{cat}}$. This early attack within the reaction coordinate for the overall rate-determining step would be expected to again generate substantial positive charge at carbon 5. In this case, hyperconjugation from the hydrogen on carbon 4 would result in the observed strong KIE upon $k_{\text{cat}}$ from the 4-deutero substrate. In addition, steric crowding of carbon 1 in the transition state would account for the inverse effect of deuterium at this same carbon. The development of positive charge at carbon 5 also accounts for the small negative slope in the linear free-energy relationship obtained previously and the rate-retarding effects of heteroatom substitutions at carbon 1 (13). The flat linear free-energy relationship plot obtained for
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$k_{cat}/K_m$ values would also be expected if the carbon 1 to endocyclic oxygen bond breaking were sufficiently advanced in the transition state of the first step.

However, the location and alignment of the unassigned aspartate are potential problems for this mechanism, because the residue is 4.01 Å from carbon 1 in the substrate-bound crystal structure of UGL from Bacillus sp. GL1 or 4.48 Å in the structure from Streptococcus agalactiae, and it is not well aligned for nucleophilic attack to displace the endocyclic oxygen. This poor positioning for nucleophilic attack at the anomeric center was noted by the authors of the substrate-bound crystal structure of UGL and indeed informed the initial formulation of the hydration-initiated mechanism. However, it is possible that either this residue or the substrate, or both, change conformation during the course of the reaction.

Another possible mechanism, which better accounts for the placement of the unassigned aspartate, involves this aspartate acting as an acid/base catalyst to activate the hydroxyl group at carbon 2 for nucleophilic attack, as shown in Fig. 5B. This oxygen can attack at carbon 1, forming a carbon 1-carbon 2 epoxide, and open the pyranose ring through breaking of the carbon 1 to endocyclic oxygen bond. This forms a ketone at carbon 5 to stabilize its developing positive charge, similar to the first mechanism proposed above. The distance between the unassigned aspartate and the carbon 2 hydroxyl group appears optimal for such a role, at 2.34 Å in the crystal structure of UGL from Bacillus sp. GL1 or 2.62 Å in that from S. agalactiae, and the carbon 2 hydroxyl itself is located anti-periplanar to the endocyclic oxygen. In a subsequent step the ketone intermediate can be hydrated and the pyranose ring re-formed, with opening of the epoxide driven by re-protonation from the unassigned aspartate.

This second mechanism accounts for all of the KIEs in the same way as the first, and it also accounts for the lack of efficient turnover of the 2-deoxy-2-fluoro substrate. Both steps in this mechanism are driven by protonation events (at carbon 4 and the epoxide), consistent with the observed solvent KIEs. Although the epoxide intermediate postulated in this mechanism would be expected to be unstable in water (35, 36), its lifetime in the enzyme active site does not need to be long, and it is still much more stable than a fully developed carbocation. Indeed, to be able to provide catalysis, it should not be too stable to avoid acting as a kinetic “sink.”

Some precedent for such a mechanism exists in nonenzymatic carbohydrate chemistry (37). In the nonenzymatic acido-catalyzed hydrolysis of glycosides, ring-opening mechanisms for glycoside hydrolysis have previously been suggested. Initial protonation can occur on either the anomeric oxygen to give a cyclic oxocarbenium ion intermediate stabilized by the endocyclic oxygen or on the endocyclic oxygen to give an acyclic oxocarbenium ion stabilized by the anomeric oxygen. Distinguishing between these two mechanisms has been the topic of many publications, and a mechanism involving protonation of the endocyclic oxygen was long under consideration for glycoside hydrolases (38). Some rearrangements, for example thiglycosides in aqueous acid (39) or constrained systems in the presence of strong Lewis acids (40), do in fact appear to proceed through such acyclic intermediates (Fig. 6A). Furthermore, the involvement of epoxide intermediates has been proposed to accelerate nonenzymatic decomposition of activated glycosides with a 1,2-trans arrangement under basic conditions (Fig. 6B) (41, 42), and nonenzymatic opening of epoxides in a hexose is accelerated by participation of a nearby ester substituent (Fig. 6C) (43–45). In another example, the reaction sequence of an exogenous nucleophile attacking at a carbonyl carbon to form the nucleophile that opens an epoxide, at the same time reforming the ring of a carbohydrate, has also been previously proposed, albeit forming a furanoside and being driven by base rather than acid catalysis (Fig. 6D) (46, 47). As final examples, 1,2-epoxides have been proposed as intermediates in glycosidase catalysis previously, although always for substrates in the pyranosyl configuration. Examples include E. coli lacZ β-galactosidase (shown not to follow such a mechanism) and a bacterial endo-mannosidase (48, 49).

Although the two mechanisms outlined above are regarded as the best candidates by the authors, other mechanisms have been considered, as well as other factors that may contribute to catalysis. A mechanism involving attack of the unassigned carboxylate directly at the site of initial charge development on carbon 5 was ruled unlikely, both because of the poor placement of this group relative to carbon 5 in the substrate-bound crystal structure (10) as well as the KIE arising from deuterium for hydrogen substitution at C1 suggesting involvement of this center in the rate-determining step of the reaction. Attack of the C2 hydroxyl group at C5 to form a bridged intermediate was also ruled unlikely on the basis of crystallographic evidence against the adoption of an appropriate conformation in the enzyme active site, despite precedence also existing for such...
mechanisms in the nonenzymatic literature (50, 51). Furthermore, such a mechanism would not explain the KIE from deuterium for hydrogen substitution at C1. Some rate enhancement may come from the C6 carboxylate, either by formation of a transient \( \alpha \)-lactone through quenching of the positive charge at C5 or by electrostatic interaction with charge at the endocyclic oxygen. Finally, during the departure of the unassigned carboxylate or its acid catalysis of epoxide opening, some degree of transient charge development may occur at carbon 1, which could be stabilized by electron donation from the anomeric oxygen. The involvement of this atom in the overall rate-determining step may explain the comparatively low turnover of the C-glycoside substrate (11), and it may also be a factor in the very low turnover of the glycosyl fluoride substrate (13). However, the thioglycoside substrate (11) was reported to cleave rapidly, despite carbon and sulfur having very similar electronegativities and sulfur lone pairs being poor electron donors to vacant \( p \)-orbitals on carbon.

**5-Fluoro Product Analogs**—Evidence for the formation of a covalent glycosyl enzyme intermediate, proposed in the first mechanism (Fig. 5A), was sought in an attempted trapping of such an intermediate using an analog of the hydrated intermediate in which fluorine replaces the hydroxyl at C5. If the mechanism in Fig. 5A is followed, such a derivative might be sufficiently reactive to undergo the “retro” reaction shown in Fig. 7, in which loss of the fluoride leaving group leads to formation of the putative acyclic glycosyl-enzyme intermediate via the reverse of its normal reaction. However a simple 5-fluoro analog would be hydrolytically unstable, as probably would the covalent glycosyl enzyme species sought. To solve this stability problem, a second fluorine is incorporated to stabilize the “anomeric” fluoride at carbon 5 through inductive destabilization of the oxocarbenium ion-like transition state for its decomposition. Two such compounds were synthesized, one in which the second fluorine is incorporated at carbon 4 (2,3-difluoro-Kdn) and one in which the second fluorine is incorporated at carbon 1 (4-deoxy-1,5-difluoro-iduronic acid), as shown in Fig. 7. These second fluorine atoms, on carbons 4 and 1, are located adjacent to sites of positive charge development (carbon 5 and oxygen 5, respectively); thus, in both cases departure of the leaving group fluoride will be slowed and the compound stabilized. An added advantage of incorporating the fluorine at carbon 4 is that, should the intermediate be formed, one of the pathways for its decomposition (elimination) is blocked, as shown in Fig. 7.

Neither of these compounds showed concentration- and time-dependent inactivation of UGL, as shown by Fig. 8, nor were they hydrolyzed by UGL at rates above their relatively high background rates (data not shown). 4-Deoxy-1,5-difluoro-iduronic acid was tested and found to be a competitive inhibitor of UGL (\( K_i \) of 7.5 ± 0.8 mM), demonstrating binding to the enzyme active site. 2,3-Difluoro-Kdn showed a similar nonspecific inactivation effect to that which was observed with the anomeric difluoride substrate analog (see above) when tested at concentrations above 20 mM. Subsequent testing showed this effect could also be observed with similar concentrations of axial \( \Delta \text{GlcUA} \) fluoride and phenyl \( \Delta \text{GlcUA} \), with this inactivation likely being the source of deviation from Michaelis-Menten kinetics noticed at high concentrations of the latter compound (11). Neither of these compounds are anticipated to be mechanism-based inactivators, further suggesting that this inactivation is a nonspecific effect. This lack of mechanism-based inactivation is evidence against any covalent glycosyl-enzyme intermediate, such as the first mechanism proposed above, although it is not conclusive. It remains possible that the fluorine at carbon 5 is not sufficiently activating to force the reverse of the hydration reaction, in particular because this fluorine is not directly displaced in the
proposed mechanisms, which first require breaking of the unactivated endocyclic oxygen to carbon 1 bond.

**Attempted Rescue of D113G**—As a further test of the mechanistic role of the unassigned aspartate, aspartate 113 was mutated to glycine in *C. perfringens* UGL. This mutation creates a large space in the enzyme active site, which can potentially be filled by exogenous nucleophiles to replace the activity of the missing side chain and recover the enzyme’s catalytic activity, as has been done successfully in the identification of active site carboxylates in conventional Koshland glycosidases (52).

The catalytic activity of the mutant D113G UGL was substantially lower than that of the wild-type UGL, but substrate bound with largely unchanged affinity as follows: $k_{cat}$ of 0.0033 ± 0.0001 s$^{-1}$ and $K_m$ of 0.8 ± 0.1 mM for 3-nitrophenyl GlcUA (cf. 14.7 ± 0.5 and 0.88 ± 0.03 mM for wild type). A conservative mutation of the analogous position in *Bacillus* sp. GL1 UGL to asparagine has previously been reported to result in similar changes in kinetic parameters (12). The catalytic activity measured here is on the same order as that which could arise from mis-incorporation of Glu at this position through a near cognate codon, resulting in “contaminating” by wild-type enzyme (53). This means that the activity measured is in fact an upper limit for the actual activity of the mutant.

Rescue of catalytic activity in this mutant was attempted with a variety of small molecules but was unsuccessful. No enhancement of UGL-catalyzed hydrolysis of 4-nitrophenyl GlcUA was seen by UV-visible spectrophotometry upon addition of sodium azide or sodium formate, at up to 100 mM concentration. Rescue of catalytic activity in this mutant was attempted with a variety of small molecules but was unsuccessful. No enhancement of UGL-catalyzed hydrolysis of 4-nitrophenyl GlcUA was seen by UV-visible spectrophotometry upon addition of sodium azide or sodium formate, at up to 100 mM concentration of either small molecule. Further testing of a wider range of nucleophiles by TLC analysis of reactions over several days also showed no rescue or signs of alternative product formation. The potential nucleophiles tested by TLC were sodium formate, sodium acetate, methanol, $\beta$-mercaptoethanol, sodium azide, sodium cyanate, sodium thiocyanate, and sodium cyanide. This absence of rescue of the UGL D113G mutant catalytic activity by exogenous nucleophiles again provides (nonconclusive) evidence against the mechanism shown in Fig. 5A, although not necessarily in support of that in Fig. 5B.

Overall, a number of experiments were presented here that provide further evidence against a simple one-step direct hydration mechanism for UGL. In an attempt to explain these results, as well as several anomalous results in earlier publications, two modified mechanisms are proposed here. These involve either a covalent glycosyl-enzyme intermediate or a noncovalent epoxide intermediate in the hydration reaction. These modified mechanisms also postulate roles for a second catalytically critical aspartate residue in the UGL active site, which does not have a defined role in the one-step hydration mechanism. In the modified mechanisms, this residue acts as either a nucleophile or an acid/base catalyst to stabilize the positive charge developing at carbon 5 of the substrate during protonation at carbon 4. Of these two mechanisms, the one involving an epoxide intermediate currently appears to be the most likely, based on the location of catalytic residues in the enzyme active site, the poor turnover of a 2-deoxy-2-fluoro substrate, and unsuccessful attempts at confirmation of a covalent glycosyl-enzyme intermediate. Although these mechanisms still await direct experimental confirmation, they provide a framework for explaining a growing body of results not consistent with the currently accepted direct hydration mechanism.

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