The α-Glucuronidase, GlcA67A, of Cellvibrio japonicus Utilizes the Carboxylate and Methyl Groups of Aldobiouronic Acid as Important Substrate Recognition Determinants*

Tibor Nagy‡, Didier Nurizzo§, Gideon J. Davies‡, Peter Biely†, Jeremy H. Lakey‡, David N. Bolam‡, and Harry J. Gilbert‡**

From the §School of Cell and Molecular Biosciences, The University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, United Kingdom, the §Department of Chemistry, Structural Biology Laboratory, The University of York, Heslington, York YO10 5DD, United Kingdom, and the ‡Institute of Chemistry, Slovak Academy of Sciences, Dubravska Cesta 9, 84328 Bratislava, Slovakia

α-Glucuronidases are key components of the ensemble of enzymes that degrade the plant cell wall. They hydrolyze the α1,2-glycosidic bond between 4-O-methyl-D-glucuronic acid (4-O-MeGlcA) and the xylan or xylooligosaccharide backbone. Here we report the crystal structure of an inactive mutant (E292A) of the α-glucuronidase, GlcA67A, from Cellvibrio japonicus in complex with its substrate. The data show that the 4-O-methyl group of the substrate is accommodated within a hydrophobic sheath flanked by Val-210 and Trp-160, whereas the carboxylate moiety is located within a positively charged region of the substrate-binding pocket. The carboxylate side chains of Glu-393 and Asp-365, on the “β-face” of 4-O-MeGlcA, form hydrogen bonds with a water molecule that is perfectly positioned to mount a nucleophilic attack at the anomeric carbon of the target glycosidic bond, providing further support for the view that, singly or together, these amino acids function as the catalytic base. The capacity of reaction products and product analogues to inhibit GlcA67A shows that the 4-O-methyl group, the carboxylate, and the xylose sugar of aldobiouronic acid all play an important role in substrate binding. Site-directed mutagenesis informed by the crystal structure of enzyme-ligand complexes was used to probe the importance of highly conserved residues at the active site of GlcA67A. The biochemical properties of K288A, R325A, and K360A show that a constellation of three basic amino acids (Lys-288, Arg-325, and Lys-360) plays a critical role in binding the carboxylate moiety of 4-O-MeGlcA. Disruption of the apolar nature of this β-arabinofuranosyl, acetyl- and 4-O-methyl-D-glucuronoyl substituents (2). The extent and composition of these “side chains” varies between plant species and the extent of cellular differentiation (2).

The microbial hydrolysis of xylans is mediated by the action of an ensemble of different enzymes. The substrates are removed by a repertoire of glycoside hydrolases and carbohydrate esterases including arabinofuranosidases, α-glucuronidases, and acetyl-xylan esterases, (2), whereas the xylose-containing polymeric backbone is hydrolyzed by endo-β-1,4-xylanases (2). Although the endo-acting xylanases have been extensively characterized, enzymes that remove the side chains have received less attention. Recent studies, however, have started to unravel the mechanism of action of α-glucuronidases.

All the α-glucuronidases characterized to date are located in glycoside hydrolase family (GH) 67, in the Henrissat classification of carbohydrate active enzymes (see Refs. 3 and 4). The majority are either membrane-bound or intracellular (5), and hydrolyze the α1,2-glycosidic bond between 4-O-methyl-D-glucuronic acid (4-O-MeGlcA) and the xylose moiety located at the non-reducing end of xylooligosaccharides (6). These enzymes catalyze bond cleavage by an S_N2-like single displacement mechanism in which water attacks the anomic carbon of the uronic acid, concomitant with protonation of the glycosidic oxygen and leaving group departure, leading to inversion of anomeric configuration and the generation of the β-anomer of 4-O-MeGlcA as product (7). Such a reaction involves both general acid assistance to leaving group departure and general base-catalyzed activation of the attacking water molecule (8).

α-Glucuronidases are the only hemieellulases that hydrolyze sugar polymers containing functional groups other than hydroxyl moieties. The extent to which these enzymes exploit the carboxylate and methyl groups of 4-O-MeGlcA in substrate recognition is largely unknown. Recently the first three-dimen-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: European Synchrotron Radiation Facility, BP 220, F-38043 Grenoble Cedex, France.

‡ To whom correspondence should be addressed. Tel.: 44-0-191-2229682; Fax: 44-0-191-2226884; E-mail: H.J.Gilbert@Newcastle.ac.uk.
α-Glucuronidase, Mechanism of Substrate Recognition

α-glucuronidase, the enzyme, GlcA67A, from *C. japonicus* (previously known as *Pseudomonas cellulosa*) is a dimeric protein that comprises three domains. The N-terminal domain forms a two-layer β-sandwich, whereas the C-terminal domain consists mainly of long α-helices that form the dimer interface. The central domain is a classical (βα)_{8} barrel that houses the catalytic apparatus. The structure of GlcA67A in complex with its reaction products implicated Glu-292 as the catalytic acid. Identification of the catalytic Basicity base, however, was ambiguous (9).

The authors describe the structure of a catalytically inactive mutant of GlcA67A (E292A) in complex with its substrate aldobiose, the affinity of the enzyme for its reaction products, and the kinetic dissection of a series of active site mutants. The structural data show that both Glu-393 and Asp-365 are appropriately positioned to provide base catalysis.

### EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, and Culture Conditions**—The *Escherichia coli* strains used in this study were TUNER (DE3) obtained from Novagen and XL1 Blue. The plasmid employed in this work, pET22, which expresses the mature form of GlcA67A fused to the C terminus of (His)_{10}-thioredoxin (5). To produce recombinant GlcA67A, *E. coli* TUNER (DE3) harboring pET21 was cultured as described previously (5).

### Recombinant DNA Techniques—DNA manipulations were carried out essentially as described by Sambrook et al. (10), and the method used to sequence DNA was detailed by Nagy et al. (5). Site-directed mutagenesis of the GlcA67A gene (*gcaA*67A) was performed using the QuikChange kit (Stratagene) employing the following primers: W160A, 5'-gtaacactccgataattacggctgtt-3'; V10A, 5'-attaataaaggaagcgcagctg-3'; V210A, 5'-attaataaatgctgaagctgtc-3'; V310A, 5'-gaggttatccgcatggttaacaacaga-3'; V3210, 5'-gaggtatatccgcatggttaacaacaga-3'; W543A, 5'-gtggaaggtatatccgcatggttaacaacaga-3'; K288A, 5'-agggttgtcggtctggtctgctg-3'; E292A, 5'-atcaataatgcgaacgccgacccgcgtg-3'; V210G, 5'-gcgggtcggcgttcgcatggttaacaacaga-3'; V210A, 5'-gcgggtcggcgttcgcatggttaacaacaga-3'.

### Purification of Proteins and Enzyme Assays—Recombinant proteins were purified in *E. coli* TUNER (DE3) harboring pET21. Glucuronidase activity was assayed in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mg/ml bovine serum albumin, at 37 °C using the human 4-nitrophenyl-β-D-glucuronide substrate. Assays were performed in a 0.01-cm path length quartz cuvette using an ADSC Quantum 4 CCD detector. A single crystal of the E292A mutant of GlcA67A in complex with aldobiose was flash-cooled (11). The crystal was then washed with 100 mM of water before uronic acid was eluted using 100 mM ammonium acetate. Fractions (1 ml) were collected and the amount of product was quantified by Dionex high performance liquid chromatography as described by Nagy et al. (5). To determine the gross fold of wild-type and mutant forms of GlcA67A, proteins were subjected to circular dichroism spectroscopy using a Jasco J-810 spectropolarimeter. The spectra were obtained at a protein concentration of 500 μM in 20 mM MES buffer, pH 7.0, at 25 °C using a 0.01-mm path length quartz cuvette (Hellma). One spectrum was the accumulation of 5 scans between 195 and 250 nm at a scan rate of 50 nm/min, the bandwidth was 2 nm.

### Structure Solution and Refinement—The E292A mutant of GlcA67A was crystallized as described previously (9) and soaked with aldobiose. Data, at 100 K, were collected on beamline ID14-4 at the European Synchrotron Radiation Facility, Grenoble, France, using an ADSC Quantum 4 CCD detector. A single crystal of the E292A mutant of GlcA67A in complex with aldobiose was flash-cooled and indexed and measured in the laboratory. Crystals belong to triclinic space group *P*, with unit cell dimensions *a* = 68.4 Å, *b* = 74.4 Å, *c* = 87.5 Å, *α* = 115.1°, *β* = 93.0°, and *γ* = 109.4° and have two molecules in the asymmetric unit. A total of 500 images of 0.5° were collected in a unique orientation. Data were processed and scaled using the HKL suite of programs (13). Data collection and final refinement statistics are given in Table I.

### RESULTS

### Crystal Structure of E292A with Aldobiose Acid—The three-dimensional structure of wild-type GlcA67A in complex with the reaction product xylose and/or the product analogue of GlcA was observed as its 2'-configured substrate. To study substrate, as opposed to product, recognition the structure of the catalytic acid mutant E292A was determined in complex with the substrate aldobiose acid at 1.5-A resolution (Table I). Electron density is clear for the 4-O-MeGal moiety and the α-glycosidic linkage in this complex (Fig. 1). In contrast to the product complexes described previously, however, no strong
density reflecting the xylosyl moiety is observed and this aglycone is presumed to be disordered. Compared with the product complex with GlcA, the pyranoside ring of the unhydrolyzed 4-O-MeGlcA likewise in C4 chair conformation, is rotated by −27° (Fig. 2). Indeed, the location of the substrate, compared with product, yields sufficient space to observe the putative attacking water molecule perfectly poised for “in-line” inverting attack at C-1. A single solvent molecule lies, as expected, in van der Waals contact, 3.2 Å from C-1 with an OW-C1-O1 angle of −165°. It is the absence of this water in the β-anomeric product complex described previously that allowed the product to swivel “upwards” to interact directly with the base pair of Asp-365 and Glu-393.

The α conformation of the glycosidic linkage in 4-O-MeGlcA supports the view that Asp-365 and/or Glu-393 act as the base catalyst; but one is not able to state with any certainty which one of the pair functions as the base, if indeed one of them acts singly in this respect. Both the OD-1 atom of Asp-365 and the OE-1 atom of Glu-393 lie 2.6 Å from the putative attacking water and both display appropriate geometry to abstract a proton from their “anti” lone pair. The OD-1 of Asp-365 also lies 2.6 Å from the NZ amine of Lys-360, which may be an unusual environment for a group to act as a base catalyst. Substituting either Asp-365 or Glu-393 with alanine rendered GlcA67A completely inactive, whereas the mutant E393C displays substantial activity leading to the suggestion that the aspartate functions as the catalytic base (9). This proposal, however, must be treated with caution as the equivalent mutation to E393C in other α-glucuronidases led to a substantial reduction in catalytic activity (16). Given the additional ambiguity of interpreting single substitutions of these carboxylates, one can only conclude that one or both contribute to base catalysis adding to the emerging picture that inverting glycosidases are rarely as simple to dissect as schematic pictures (typical of half the reaction coordinate in which the re-protonation and de-protonation of the catalytic acid and base, respectively, are not shown) suggest (17).

As described above, it is perhaps surprising that the xylosyl moiety is not readily apparent in the substrate-enzyme complex, nor is the loop between residues Phe-327 and Glu-334. Consistent with these observations is the more “open” position of the tryptophan “drawbridge” provided by Trp-543 that should stack with the aglycone xyloside, and indeed does so in the product GlcA-Xyl complex of wild-type enzyme with β-GlcA in the −1 subsite and Xyl at +1. One may only speculate as to the origin of these observations, but the loss of the binding energy provided by the catalytic acid Glu-292 in the wild-type enzyme may be a contributing factor. Furthermore, the catalytic apparatus is evolved to stabilize a dissociated oxocarbonium ion-like transition state in which the C1-OR bond is quite extended (reflected in Brønsted $p_K_a$ values tending toward −1 measured on analogous systems (18) and not to provide optimal binding for a substrate complex with a 1.4-Å C1-OR bond. The 1500-fold decrease in catalytic efficiency displayed by the W543A mutant enzyme, described below, suggests that it is contributing greatly to catalysis, even if it is not bound to an ordered xylosyl moiety in the mutant x-ray structure.

Characterization of GlcA67A Mutants—The structure of GlcA67A in complex with a range of ligands reveals the amino acids that are likely to play a key role in substrate binding and catalysis. Site-directed variants of the invariant amino acids (Fig. 3) that interact with the carboxylate and methyl moieties of 4-O-MeGlcA and the aromatic residues that stack against the sugar rings in the substrate aldobiouronic acid were therefore characterized. Each of the mutants was purified to electrophoretic homogeneity (Fig. 4). Circular dichroism spectra, recorded for all the mutants that display low activity (except E292A for which a crystal structure is available), were indistinguishable from wild-type enzyme indicating that the mutations did not significantly disrupt the protein fold (Fig. 5).

Amino Acids That Interact with the Carboxylate Group of the Substrate—Purified enzymes were incubated with 4-nitrophenyl-2-O-(4-O-methyl-α-D-glucuronosyl)-β-D-xylopyranoside and the release of 4-nitrophenol was monitored spectrophotometrically. The data (Table II) show that removal of the residues that neutralize the negative charge of the uronic acid caused a substantial reduction in the catalytic efficiency of the enzyme. The individual kinetic parameters of R325A could not be determined as there was a linear relationship between catalytic rate and the concentration of substrate up to 15 mM. The catalytic efficiency of R325A, however, was −10-fold less active than the wild-type enzyme. Substitution of the two lysine residues with alanine resulted in an increase in $K_m$ by a very large reduction in $k_{cat}$. The catalytic efficiency of K288A and K360A were 30- and 200-fold higher, respectively than the arginine mutant.

The observation that R325A displayed considerably lower activity than the two lysine mutants is consistent with the location of the guanidinium group of Arg-325, which is ideally positioned to form strong hydrogen bonds with both oxygens of the carboxyl group of 4-O-MeGlcA. It is interesting to note that although the R325A mutant retains the capacity to form two hydrogen bonds with the carboxyl group of the substrate, through Lys-288 and Lys-360, the loss of the guanidino group has a profound effect on the capacity of the enzyme to bind the uronic acid. This is consistent with the geometry of a carboxylate and a guanidino moiety that are highly complementary, and thus the direction and co-operativity of the H-bonds leads to a very tight interaction between these two functional groups, as has been witnessed many times previously (19). Furthermore, the electrostatic interaction of the carboxylate with the guanidino group is likely to be stronger than with the ε-NZ moiety of lysine as the solution $p_K_a$ values of arginine and lysine are 12.5 and 10.8, respectively (20). Whereas both lysine residues interact with the carboxylate moiety of 4-O-MeGlcA, Lys-288 also forms an H-bond with the C4 oxygen of the uronic acid and thus may play a more important role in substrate binding than Lys-360, consistent with the lower activity of K288A compared with K360A.

| Table I |
|---|
| Data and structure quality statistics for the E292A mutant of GlcA67A in complex with aldobiouronic acid |
| Data | GlcA67A mutant E292A in complex with aldobiouronic acid |
| Radiation source | ESRF, ID14-4 |
| Wavelength (Å) | 0.933 |
| Resolution (Å) | 20.0–1.50 (1.53–1.50) |
| Unique reflections | 212953 (8276) |
| Completeness (%) | 91.2 (52.9) |
| $R_{int}$ (%) | 4.7 (23.7) |
| Multiplicity | 2.7 (2.6) |
| $I/|I|$ | 20.0 (4.2) |
| $R_{cryst}$ (%) | 11.8 |
| $R_{free}$ (%) | 15.3 |
| Root mean square on bond distances (Å) | 0.017 |
| Root mean square on bond angles (°) | 1.396 |
| Residues | 1414 |
| Water | 962 |
| Heteroatoms | 2 |
| Substrate | 10 |
| Cobalt | 25 |

---

α-Glucuronidase, Mechanism of Substrate Recognition

---
Amino Acids Involved in Binding the Pyranoside Rings of the Substrate—Two aromatic amino acids Trp-160 and Trp-543 form hydrophobic “stacking” interactions with the sugar rings of 4-O-MeGlcA and xylose (9), respectively. Replacing either tryptophan residue with alanine resulted in a large decrease in catalytic efficiency. The $k_{cat}$ displayed by W160A was approximately 4-fold lower than W543A, indicating that the stacking interaction between Trp-160 and 4-O-MeGlcA plays a more significant role in catalysis than the hydrophobic platform provided by Trp-543 for the aglycone sugar. Furthermore, the W160A mutation caused a larger increase in $K_M$ than the W543A substitution. This is consistent with the prediction that the interaction of GlcA67A with the xylosyl residue, via Trp-543, would be relatively weak, as diffusion of 4-O-MeGlcA out of the substrate-binding pocket after glycosidic bond cleavage requires prior departure of the xylose residue from the active site. Indeed retaining glycoside hydrolyses have been shown to bind weakly to the aglycone region of the substrate, and reducing this interaction further can cause a substantial increase in maximum catalytic activity, although there is a concomitant elevation in $K_M$ (21, 22). Bronnenmeier et al. (6), however, showed that 4-nitrophenyl-$\alpha$-glucuronic acid was not hydrolyzed by $\alpha$-glucuronidases, which is in sharp contrast to retaining glycoside hydrolyses that are generally able to accommodate aryl groups in the +1 subsite (23, 24) pointing to the importance of sugar binding in the +1 subsite in $\alpha$-glucuronidases. This interpretation of the study by Bronnenmeier (6) should, however, be treated with some caution, as the lack of activity against the aryl-$\alpha$-glucuronide may be because of the absence of the 4-O-methyl group and not the aglycone sugar.

Methoxy Group Specificity—Together, the aliphatic residue Val-210, combined with Trp-160, form a hydrophobic sheath that accommodates the methyl moiety of 4-O-MeGlcA. To evaluate the importance of Val-210 in substrate binding, the residue was replaced with both polar and non-polar amino acids. The data show that complete removal of the valine side chain (V210G) had little influence on the catalytic activity of GlcA67A, similarly, replacing Val-210 with a smaller aliphatic amino acid by introducing the mutation V210A had no significant effect on the kinetic parameters of the glycoside hydrolase. In contrast, replacing Val-210 with more polar amino acids was detrimental to the catalytic activity of the $\alpha$-glucuronidase. Thus, both V210N and V210S were considerably less active than the wild-type enzyme. Although the individual kinetic parameters of V210N and V210S could not be determined, a linear relationship was observed between catalytic rate and substrate concentration up to 15 mM, indicating that the $K_M$ value is $\geq 15$ mM, and thus the $k_{cat}$ values of the two mutants are likely to be similar to the wild-type enzyme. It is interesting to note that although Val-210 could be substituted for a glycine or alanine residue without causing a significant change in the catalytic properties of GlcA67A, the residue is completely conserved in GH67 $\alpha$-glucuronidases. Thus, the evolutionary rationale for the conservation of this amino acid is currently unclear.

The Kinetics of Enzyme Inhibition—To evaluate the importance of the functional groups of aldoobiouroninc in substrate recognition by GlcA67A, the capacity of reaction products and their analogues to inhibit the activity of the $\alpha$-glucuronidase was determined. The full data set, presented in Table III, show that 4-O-MeGlcA is a more effective inhibitor than GlcA demonstrating that the 4-methyl group of the uronic acid makes a significant contribution to binding. The importance of the 4-methyl group in substrate binding, however, may not be a general feature of $\alpha$-glucuronidases. Indeed, Uchida et al. (25) showed that an intracellular Aspergillus niger $\alpha$-glucuronidase released GlcA moieties from aldouronic acids faster than 4-O-MeGlcA. The high $K_I$ for glucose, compared with GlcA, demonstrates that the carboxylate moiety also plays an important role in enzyme binding, which is supported by the observation that the methyl ester of the substrate 4-nitropheny1-2-O-(4-O-methyl-$\alpha$-d-glucuronosyl)-$\beta$-d-xylopyranoside is not hydrolyzed by GlcA67A.

The $K_I$ values of GlcA for the mutants V210N and V210S were considerably higher than for the wild-type enzyme, similar to the effect these mutations had on the $K_M$ for the substrate. Thus the increase in the polarity of the region of GlcA67A occupied by Val-210 does not reduce the affinity for the substrate solely by influencing the capacity of the enzyme to accommodate the methyl group of aldoobiouronic acid. It is possible that the increase in polarity caused a subtle change in the position of Trp-160, an amino acid that plays a key role in binding the sugar ring of 4-O-MeGlcA.
The high $K_i$ of xylose for GlcA67A demonstrates that the aglycone sugar interacts weakly with the enzyme. This is entirely consistent with the mechanism of product departure from the active site of the $\beta$-glucuronidase. Thus, after bond cleavage, xylose must be released from the enzyme prior to 4-O-MeGlcA as the aglycone sugar blocks departure of the uronic acid from the substrate-binding pocket. It should be emphasized, however, that the binding of the xylose component of the substrate to GlcA67A plays an important role in catalysis as the loss of the aglycone binding site through the W453A mutation caused a substantial decrease in catalytic activity (Table II). The observation that removal of Trp-543 had a detrimental effect on xylose binding is evidenced by the observation that the pentaose sugar displayed no apparent affinity for the mutant enzyme W543A. The weak binding of xylose at the aglycone binding site may explain why the sugar appeared to be disordered in the aldobiouronic acid-GlcA67A complex (Fig. 1). Thus, although 4-O-MeGlcA occupied the binding site pocket in all the enzymes in the crystal, xylose interacted with only a small number of the protein molecules. The detection of xylose in the active site in our previous study (9) likely reflects the very high level of sugar used to soak the crystals. Furthermore, it is interesting to note that when the enzyme is incubated with a high concentration of GlcA, the $K_i$ value for xylose does not decrease. Thus the effect of the uronic acid in stabilizing the location of Trp-543 (9) does not influence sugar binding at the aglycone region of the active site.

**DISCUSSION**

The crystal structure of the catalytic acid mutant of GlcA67A, E292A, in complex with aldobioseuronic acid provides the first glimpses of the true substrate of the enzyme bound at the active site and allows more precise description of the crucial $\alpha$ subsite interactions than was possible with the pseudoproduct complexes described earlier. The structure reveals, for the first time, that the 4-O-methyl group is located within a


hydrophobic sheath formed by Val-210 and Trp-160, with the O-4 atom identically located in both GlcA and 4-O-MeGlcA complexes. The difference in orientation of GlcA with the 4-O-MeGlcA described here is attributable to the appropriately positioned attacking water molecule in this complex together with the α-linked C1-OR bond of the substrate, as opposed to the β-linkage of the product. It is possible that the +1 subsite xylosyl moiety is disordered, as it displays much lower affinity for the enzyme that 4-O-MeGlcA; it is unlikely that this is because of a low residual catalytic activity (below detection by high performance liquid chromatography) because 4-O-MeGlcA is found entirely as its α-anomer, consistent with the α1,2-linkage between the uronic acid and xylose, whereas only the β-anomer of GlcA bound to the wild-type α-glucuronidase. 

The observation that W543A is more active than W160A likely reflects the importance of the interactions of the enzyme with the glucose sugar 4-O-MeGlcA, mediated in part by Trp-160, compared with the aglycone xylosyl saccharide. To achieve glycosidic bond cleavage the uronic acid must be distorted from its relaxed 4C1 chair conformation into a geometry that can accommodate the oxocarbenium ion-like transition state. Thus Trp-160 would appear not only to play an important role in binding the uronic acid in its ground state (4C1 chair) but also in stabilizing the sugar when it is distorted into its transition state conformation. Similarly, the interaction of the enzyme with the carbonylate group through Lys-288, Lys-360, and Arg-325 (inferred) are likely to also play a pivotal role in transition state stabilization, as removal of these residues reduces \( k_{cat} \) significantly. It should be emphasized, however, that as the \( k_{cat} \) of W543A is significantly lower than wild-type GlcA67A, the energy derived from the hydrophobic stacking interaction between the xylosyl residue and Trp-543 does contribute to transition state stabilization.

Although the reduction in \( k_{cat} \) displayed by mutants lacking either the three basic or two aromatic residues may reflect a loss in transition state stabilization, a decrease in catalytic activity can also be caused by a change in the position and/or \( p_K \) of the catalytic residues, or a shift in the location of the substrate within the active site such that the glycosidic oxygen and/or and the anomeric carbon is located further away from the catalytic acid and activated water molecule, respectively. Inspection of the crystal structure of the GlcA67A complexes reveals that Trp-160, Val-210, Arg-325, and Trp-543 do not form direct hydrogen bonds with the proposed catalytic residues indicating that mutations of these amino acids are unlikely to influence the function of either the Brønsted base or acid. It is not possible, however, to rule out the possibility that one or more of the mutations caused a change in the position of the substrate such that the anomic carbon or glycosidic oxygen were no longer in close proximity to the catalytic base and acid, respectively, providing an alternative explanation for the low \( k_{cat} \) values displayed by these mutants. In contrast, the NZ amine of Lys-288 and Lys-360 form H-bonds with the OE-1 of the Brønsted acid Glu-292 and the OD-1 of the putative catalytic base Asp-365, respectively. Thus, it is likely that the low \( k_{cat} \) displayed by K288A and K360A, at least in part, is because of a change in the position and/or \( p_K \) of the catalytic amino acids.

It is important to note that the Geobacillus stearothermophilus (formerly Bacillus stearothermophilus) α-glucuronidase (AguA) and GlcA67A display significant differences in their capacity to bind the reaction products. Thus binding of GlcA, xylose (in the presence of GlcA), and 4-O-MeGlcA to AguA could be measured by isothermal calorimetry, whereas in GlcA67A no significant heat was released on product binding precluding the use of isothermal calorimetry to determine affinity. Another significant difference between AguA and GlcA67A is that GlcA increased the affinity of the enzyme for xylose, consistent with good electron density for two xylose residues in the crystal structure of an inactive mutant of AguA in complex with the substrate aldotetrauronic acid (Protein Data Base accession number 1K9F), whereas in GlcA67A this potentiation in aglycone binding was not observed. Indeed the affinity of AguA for the reaction product xylotriose in the presence of GlcA was actually higher than for the substrate, which is rather counterintuitive. Furthermore, the \( K_D \) of the enzyme for its substrate and reaction products were considerably lower than its \( K_M \). It is possible that these apparent anomalies may reflect the temperature at which isothermal calorimetry and enzyme assays were performed, 30 and 55 °C, respectively.

This study shows that GlcA67A utilizes the carboxylate and methyl groups of aldobiouronic acid as important substrate recognition determinants. The interaction between these functional groups and the enzyme not only mediates tighter binding, as evidenced by an increase in the \( K_M \) of mutants when
these interactions are disrupted and the \( K_i \) values of the reaction products and their analogues, but also in catalysis (low \( k_{cat} \) values displayed by the mutants) probably by stabilizing the transition state conformation of 4-O-MeGlcA. The six residues, which have been the focus of this study, are completely conserved in GH67, the only enzyme family that is known to contain \( \alpha \)-glucuronidases. Thus, the analysis of the role of these amino acids in substrate binding and catalysis in GlcA67A is of generic relevance to all the \( \beta \)-glucuronidases described to date.

REFERENCES
1. Coughlan, M. P., and Hazlewood, G. P. (1993) Biotechnol. Appl. Biochem. 17, 259–289
2. Brett, C. T., and Waldren, K. (1996) in Physiology and Biochemistry of Plant Cell Walls: Topics in Plant Functional Biology (Black, M., and Charlewood, B., eds) Vol. 1, pp. 16–31, Chapman and Hall, London
3. Henrissat, B., and Bairoch, A. (1996) Biochem. J. 316, 695–696
4. Coutinho, P. M., and Henrissat, B. (1999) in Recent Advances in Carbohydrate Engineering (Gilbert, H. J., Davies, G. J., Henrissat, B., and Svensson, B., eds) pp. 3–12, Royal Society of Chemistry, Cambridge
5. Nagy, T., Emami, K., Ferreira, L. M., Humphry, D. R., and Gilbert, H. J. (2002) J. Bacteriol. 184, 4925–4929
6. Bronnenmeier, K., Meissner, H., Stocker, S., and Staudenbauer, W. L. (1995) Microbiology 141, 2033–2040
7. Biely, P., de Vries, R. P., Vrsanska, M., and Visser, J. (2000) Biochim. Biophys. Acta 1474, 360–364
8. Koshland, D. E. (1953) Biol. Rev. 28, 416–436
9. Nurizzo, D., Nagy, T., Gilbert, H. J., and Davies, G. J. (2002) Structure 10, 547–556
10. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
11. Biely, P., Hirsch, J., la Grange, D. C., van Zyl, W. H., and Prior, B. A. (2000) Anal. Biochem. 286, 289–294
12. Charnock, S. J., Lakey, J. H., Virden, R., Hughes, N., Sinnott, M. L., Hazlewood, G. P., Pickersgill, R., and Gilbert, H. J. (1997) J. Biol. Chem. 272, 2942–2951
13. Otwonowki, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
14. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D 53, 240–255
15. Perrakis, A., Morris, R., and Lamzin, V. S. (1999) Nat. Struct. Biol. 6, 458–463
16. Zaide, G., Shallom, D., Shulami, S., Zolotnitsky, G., Golan, G., Baoav, T., Shasham, G., and Shasham, Y. (2001) Eur. J. Biochem. 268, 3006–3016
17. Keivula, A., Ruohonen, L., Wohlfahrt, G., Reinkainen, T., Teger, T. T., Piens, K., Claeyssens, M., Weber, M., Vasella, A., Becker, D., Sinnott, M. L., Zou, J. Y., Kleywegt, G. J., Sturmenings, M., Stalsberg, J., and Jones, T. A. (2002) J. Am. Chem. Soc. 124, 10015–10024
18. Sinnott, M. L. (1998) Biochem. Soc. Trans. 26, 160–164
19. Wigley, D. B., Lyall, A., Hart, K. W., and Holbrook, J. J. (1987) Biochem. Biophys. Res. Commun. 149, 927–929
20. Strey, L. (2002) in Biochemistry, 5th Ed., pp. 50, W. H. Freeman and Co., New York
21. Andrews, S. R., Charnock, S. J., Lakey, J. H., Davies, G. J., Claeyssens, M., Nerinckx, W., Underwood, M., Sinnott, M. L., Warren, B. A., and Gilbert, H. J. (2000) J. Biol. Chem. 275, 23027–23033
22. Schmidt, A., Gohritz, G. M., and Kratky, C. (1999) Biochemistry 38, 2403–2412
23. Tull, D., and Withers, S. G. (1994) Biochemistry 33, 6363–6370
24. Charnock, S. J., Spurway, T. D., Xie, H., Beylot, M. H., Virden, R., Warren, R. A., Hazlewood, G. P., and Gilbert, H. J. (1998) J. Biol. Chem. 273, 3187–3199
25. Uchida, H., Nanri, T., Kawabata, Y., Kusakabe, I., and Murakami, K. (1992) Biosci. Biotechnol. Biochem. 56, 1608–1616
The α-Glucuronidase, GlcA67A, of Cellvibrio japonicus Utilizes the Carboxylate and Methyl Groups of Aldobiouronic Acid as Important Substrate Recognition Determinants

Tibor Nagy, Didier Nurizzo, Gideon J. Davies, Peter Biely, Jeremy H. Lakey, David N. Bolam and Harry J. Gilbert

J. Biol. Chem. 2003, 278:20286-20292.
doi: 10.1074/jbc.M302205200 originally published online March 24, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302205200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 19 references, 5 of which can be accessed free at http://www.jbc.org/content/278/22/20286.full.html#ref-list-1