Evidence That GH115 \(\alpha\)-Glucuronidase Activity, Which Is Required to Degrade Plant Biomass, Is Dependent on Conformational Flexibility*

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Artur Rogowski\(^1\), Arnaud Baslé\(^1\), Cristiane S. Farinas\(^1,5\), Alexandra Solovyova\(^1\), Jennifer C. Mortimer\(^5\), Paul Dupree\(^5\), Harry J. Gilbert\(^1,2\) and David N. Bolam\(^1,3\)

From the \(^4\)Institute for Cell and Molecular Biosciences, The Medical School, Newcastle University, Newcastle upon Tyne NE2 4HH United Kingdom and the \(^5\)Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, United Kingdom

**Background:** The structure of GH115 glucuronidas that remove glucuronic acid from xylan chains is unknown.

**Results:** *Bacteroides ovatus* GH115 glucuronidas is a dimeric enzyme that contains a flexible active site pocket.

**Conclusion:** The assembly of the catalytic apparatus of the glucuronidas requires substantial conformational changes.

**Significance:** Conformational changes are highly unusual in glycoside hydrolases.

The microbial degradation of the plant cell wall is an important biological process that is highly relevant to environmentally significant industries such as the bioenergy and biorefining sectors. A major component of the wall is glucuronoxylan, a \(1,4\)-linked xylose polysaccharide that is decorated with \(\alpha\)-linked glucuronic and/or methylglucuronic acid (GlcA/MeGlcA). Recently three members of a glycoside hydrolase family, GH115, were shown to hydroylze MeGlcA side chains from the internal regions of xylan, an activity that has not previously been described. Here we show that a dominant member of the human microbiota, *Bacteroides ovatus*, contains a GH115 enzyme, *BoAgu115A*, which displays glucuronoxylan \(\alpha-(4-O\text{-methyl})\)-glucuronidas activity. The enzyme is significantly more active against substrates in which the xylose decorated with GlcA/MeGlcA is flanked by one or more xylose residues. The crystal structure of *BoAgu115A* revealed a four-domain protein in which the active site, comprising a pocket that abuts a cleft-like structure, is housed in the second domain that adopts a TIM barrel-fold. The third domain, a five-helical bundle, and the C-terminal \(\beta\)-sandwich domain make inter-chain contacts leading to protein dimerization. Informed by the structure of the enzyme in complex with GlcA in its open ring form, in conjunction with mutagenesis studies, the potential substrate binding and catalytically significant amino acids were identified. Based on the catalytic importance of residues located on a highly flexible loop, the enzyme is required to undergo a substantial conformational change to form a productive Michaelis complex with glucuronoxylan.

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1. Present address: Embrapa Instrumentation, Rua XV de Novembro 1452, CEP 13560–970, São Carlos, SP, Brazil.

2. To whom correspondence may be addressed. E-mail: harry.gilbert@ncl.ac.uk.

3. To whom correspondence may be addressed. E-mail: david.bolam@ncl.ac.uk.
pointing at the surface of the enzymes, explaining why these \( \alpha \)-glucuronidases can only target uronic acids attached to the non-reducing end of xylan chains (10, 11). Recently, two microbial eukaryotic enzymes from GH115 were shown to be \( \alpha \)-glucuronidases that could cleave GlcA and MeGlcA from both the non-reducing end and internal xylose units of xylan and xylo-oligosaccharides (12, 13), displaying a single displacement acid base-assisted (inverting) mechanism, which results in inversion of the anomeric configuration of the cleaved uronic acid (14). The two enzymes had a higher specific activity against glucurono-xylooligosaccharides compared with glucuronoxyalan, although no kinetic parameters for these enzymes were reported. As no crystal structure for a GH115 enzyme is available, the structural basis for the specificity of these \( \alpha \)-glucuronidases is unknown.

To date, GH115 contains 237 members of which the majority (177) are bacterial, although only a single prokaryotic enzyme (from \textit{Streptomyces pristinaespiralis}) has been shown to display \( \alpha \)-glucuronidase activity (15). Bacteroidetes is a major phylum in the human gut microbiota. These organisms, exemplified by \textit{Bacteroides ovatus}, are capable of degrading an extensive range of human and plant glycans (16). This glycan-degrading capacity is orchestrated by physically linked and co-regulated genes that are known as polysaccharide utilization loci (see Ref. 17 for review). The genome of \textit{B. ovatus} contains seven open reading frames that are annotated as GH115s, two of which are in a polysaccharide utilization locus that is up-regulated during growth on complex xylans (16). Here we report the biochemical characterization and structure of one of these enzymes, BACOVA\_03449, defined henceforth as \textit{BoAgu115A}. The enzyme was shown to be a glucuronoxylan-specific \( \alpha \)-glucuronidase that displayed a strong preference for uronic acids that decorate internal xylose units. The enzyme displays the same core-fold as GH67 \( \alpha \)-glucuronidases, however, the active site residues are not conserved in the two enzymes. Mutagenesis and structural data indicate that a significant conformational change is required to assemble the catalytic apparatus in \textit{BoAgu115A}.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of GH115 Enzymes**—The open reading frame encoding mature forms (and truncated derivatives of \textit{BoAgu115A}) of the seven \textit{B. ovatus} GH115 proteins were amplified from genomic DNA (\textit{B. ovatus ATCC 8483}) by PCR using primers that introduce NheI and XhoI restriction enzyme sites. The amplified DNA was cloned into the \textit{Escherichia coli} expression vector pET28a (Novagen) such that the encoded recombinant proteins contain an N-terminal His\(_{6}\) tag. The recombinant proteins were produced in \textit{E. coli} BL21 DE3 (Novagen) cells, harboring appropriate pET-based plasmids, and cultured in LB broth containing kanamycin (50 \( \mu \)g/ml) at 37 °C. Cells were grown to mid-exponential phase (\( A_{600} \) of 0.6), at which point isopropyl \( \beta \)-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the cultures were incubated for a further 16 h at 16 °C. The cells were harvested by centrifugation, and His\(_{6}\)-tagged recombinant protein was purified from cell-free extracts by immobilized metal affinity chromatography (IMAC) using standard methodology (18). For crystallographic studies \textit{BoAg}u115A was further purified by size exclusion chromatography using a Superdex 75 column. All proteins were purified to electrophoretic homogeneity as judged by SDS-PAGE.

**Mutagenesis**—Site-directed mutagenesis was conducted using the PCR-based QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions, using the plasmid encoding \textit{BoAg}u115A as the template and appropriate primer pairs.

**Nomenclature of Oligosaccharides**—The oligosaccharides are defined as follows: for homopolymers of \( \beta \)1,4-linked xylose residues either the full name of the saccharide is used or the degree of polymerization is indicated as a subscript (\( e.g. \) the tetrasaccharide of xylose is denoted as xylotetraose or \( X_4 \)). Glucurono-xylooligosaccharides are identified by their sequence (unless otherwise stated) in which X is an undecorated xylose and U is a xylose containing an \( \alpha \)1,2-linked [Me]GlcA (\( e.g. \) UXXX is xylotetraose in which the xylose at the non-reducing end contains an \( \alpha \)1,2-linked [Me]GlcA).

**Enzyme Assays**—Substrates consisted of glucurono-xylans or glucurono-xylooligosaccharides. The glucurono-xylooligosaccharides UX and UXXX were purchased from Megazyme International (County Wicklow, Ireland), whereas UXX and XUXX were made as follows: 5 g of birch wood xylan (Sigma) was digested to completion with either \( \textit{CjXyn10A} \) (19) or \( \textit{NpXyn11A} \) (20) xylanases in 50 mM sodium phosphate, 12 mM citrate buffer, pH 6.5, containing 1 mg/ml of BSA (PC buffer) to completion. The glucurono-xylooligosaccharides generated by \( \textit{CjXyn10A} \), UXX, or \( \textit{NpXyn11A} \), XUXX, were purified from neutral xylooligosaccharides using Dowex chromatography (9). The structure of the products were confirmed by incubation with \( \textit{CjGlcA67A} \), which generated xylotriose from the \( \textit{CjXyn10A} \) product but no undecorated xylooligosaccharide from the \( \textit{NpXyn11A} \) product, whereas \textit{BoAg}u115A released xylotriose and xylotetraose from glucurono-xylooligosaccharides generated by the \( \textit{GH10} \) and \( \textit{GH11} \) xylanases, respectively. The deduced structure of the two glucurono-xylooligosaccharides is entirely consistent with the glucuronoxylan binding mode of the two xylanases (21, 22). Enzyme assays, in which polysaccharides or glucurono-xylooligosaccharides were the substrates, were carried out in PC buffer at 37 °C using enzyme purified to electrophoretic homogeneity by immobilized metal ion affinity chromatography. The concentration of enzyme varied from 10 nm for the wild type glucuronidase to 10 \( \mu \)M for the least active variants of \textit{BoAg}u115A. For kinetic assays, glucu-
ronic acid was detected using the α-D-glucoroni
dase assay kit in which the uronic acid released is oxidized to glucarate with the concomitant reduction of NAD\(^+\) to NADH, which was moni
tored continuously at 340 nm and quantified using a molar
extinction coefficient of 6220 M\(^{-1}\) cm\(^{-1}\). The molar concentra
tion of the GlcA/MeGlcA in the glucuronoxylans was quanti
cified by digesting 100 μg of the polysaccharides to completion
with BoAgu115A. To measure the activity of the BoAgu115A
mutants the xylotetraose reaction product released from
XUXX was monitored by high performance anion-exchange
chromatography (HPAEC) as described previously (18). The
reaction was carried out in 20 mM sodium phosphate buffer, pH
7.0, at a substrate concentration (1 mM) that was well below the
K\(_M\). Thus, the initial rate of hydrolysis of the glucurono-xyl-
igosaccharides gives a direct readout of k\(_{\text{cat}}$/K\(_M\) (19). The glucu-
ronoxylanc-vated reactions were also subjected to Polysac-
caride Analysis using Carbohydrate gel Electrophoresis (PACE)\(^4\) as follows: a alcohol-insoluble residue was prepared from mature Arabidopsis thaliana wild type and gus1gux2
stems as well as wild type willow, barley, sugar cane, and Miscan-
tus stems, as previously described (23). Alcohol-insoluble residue (500 μg) was pre-treated with 20 μL of 4 M NaOH for 1 h,
neutralized with HCl, and ammonium acetate buffer, pH 6.0,
added to a final concentration of 0.1 M and a final volume of 500 μL. The Arabidopsis alcohol-insoluble residue was digested to
completion with xylanases (C/Xyn10A and NpXyn11A), a
glucuronoxylanase (BoGH30; Bacova_03432), and GH67 and
GH115 α-glucuronidases (C/GlcA67A and BoAgu115A) as
stated in the text and then dried in vacuum, whereas the other xylans were just digested with BoAgu115A. Released mono-
and oligosaccharides were labeled with 8-aminonaphthalene-
Sulfonic acid was detected using the
HKL2MAP interface for the ShelxC/D/E pipeline
(27). All other computing used the CCP4 suite of programs (28).

\(^4\) The abbreviation used is: PACE, polysaccharide analysis using carbohydrate
gel electrophoresis.

### TABLE 1

**Data statistics and refinement details**

| Data statistics* | Native | Selenomethionine | Ligand |
|-----------------|--------|-----------------|-------|
| **Beafline**    | I02    | I02             | I04-1 |
| **Date**        | 18/12/10 | 27/02/11        | 16/10/11 |
| **Wavelength (Å)** | 0.9795 | 0.9795 | 0.9173 |
| **Resolution (Å)** | 65.52-2.65 | 67.05-3.00 | 2.14 |
| **Space group** | P2\(_1\)/2\(_1\)/2 | P2\(_1\)/2\(_1\)/2 | P2\(_1\)/2\(_1\)/2 |
| **Unit cell parameters** | a (Å): 75.39 | 76.13 | 72.04 |
| **Solvent content (%)** | 56 | 54 | 48 |
| **Data collection** | 219,395 | 1,240,973 | 272,938 |
| **Completeness (%)** | 97.5 (94.5) | 100.0 (100.0) | 94.4 (91.5) |
| **Redundancy** | 3.9 (3.8) | 29.8 (29.9) | 2.9 (2.7) |
| **Rmerge (%)** | 12.6 (49.7) | 19.8 (57.3) | 9.6 (42.5) |
| **Anomalous completeness (%)** | NA | 100.0 (100.0) | NA |
| **Anomalous redundancy** | NA | 15.5 (15.4) | NA |

**Refinement statistics**

| **Rfactor (%)** | 20.34 | NA | 17.40 |
| **Rfree (%)** | 26.59 | NA | 21.91 |
| **No. of non-H atoms** | 12888 | NA | 12695 |
| **No. of water molecules** | 23 | NA | 602 |
| **No. of ligand atoms** | 2 | NA | 13 |
| **Average B factor (Å\(^2\))** | 31.5 | NA | 26.9 |
| **Ligand** | NA | NA | 26.9 |
| **Solvent water** | 22.5 | NA | 24.8 |
| **Solvent (sodium)** | 26.5 | NA | 37.3 |
| **Ramachandran plot, residues in allowed and most favored regions (%)** | 99.63 | NA | 99.81 |
| **Protein Data Bank codes** | 4C90 | 4C91 | 4C91 |

* Values in parentheses are for the highest resolution shell.

**GH115 α-Glucuronidase**

BoAgu115A Is a Xylan-specific α-Glucuronidase—The genome of B. ovatus encodes seven proteins that are members of GH115.

Phases were extended to an initial native dataset at 2.65 Å and a starting model was built using Buccaneer (29). The model underwent recursive cycles of model building in COOT (30) and refinement in REFMAC (31). The native model was used as the search model for molecular replacement in Molrep to solve the ligand datasets (32). Solvent molecules were added using COOT and checked manually. Five percent of the observations were randomly selected for the R\(_{free}\) set. The models were validated using Molprobity (33). The data statistics and refinement details are reported in Table 1.

**Analytical Ultracentrifugation**—Sedimentation velocity experi-
ments were carried out in a Beckman Coulter (Palo Alto, CA)
ProteomeLab XL-I analytical ultracentrifuge using interference
optics. All analytical ultracentrifugation runs were carried out at
the rotation speed of 48,000 rpm and experimental temper-
ature of 20 °C; the velocity scans were taken 1 s apart, 600 scans
in total. The sample volume was 400 μL. The rate protein sedi-
mentation was used to calculate the M\(_c\) of the glucuronidase as
described previously (34).

**RESULTS AND DISCUSSION**

BoAgu115A Is a Xylan-specific α-Glucuronidase—The genome of B. ovatus encodes seven proteins that are members of GH115.
To explore their potential enzymatic activities, six of the seven B. ovatus GH115 proteins were expressed in E. coli in soluble form (BACOVA_00249 could not be expressed in E. coli), purified by IMAC to electrophoretic homogeneity, and their activity against glucuronoxylans was explored using PACE. The data showed that BoAgu115A released glucuronic acid (GlcA) and 4-O-methyl-GlcA (MeGlcA) from wild type Arabidopsis glucuronoxylan, but not from an Arabidopsis mutant completely lacking MeGlcA and GlcA decorations on xylan (gux1gux2) (23) (Fig. 2A). The release of both GlcA and MeGlcA demonstrate that the enzyme can accommodate the 4-O-methyl substitution of GlcA, a trait shared with the other family of \( \alpha \)-glucuronidases, GH67. The enzyme could also remove MeGlcA from glucuronoxylans present in bioenergy-relevant dicots such as willow, and from the more complex glucuronoarabinoxylans derived from monocots such as barley, sugar cane, and Miscanthus (Fig. 2B). Thus, BoAgu115A is a \( \alpha \)-glucuronidase that targets the uronic acids that decorate xylans. None of the other B. ovatus GH115 proteins exhibited measurable catalytic activity against a range of plant polysaccharides that contain uronic acids including glucuronoxylans, homogalacturonic acid, and rhamnogalacturonan I and II (data not shown). The lack of activity of one of these B. ovatus enzymes, BACOVA_00434, against glucuronoxylan is surprising as it is closely related to BoAgu115A (54% identity), and is encoded by a gene within the same xylan-activated polysaccharide utilization locus. In contrast, BACOVA_00492, BACOVA_00982, BACOVA_02173, and BACOVA_02777 are more distantly related to BoAgu115A and are not expressed in response to xylans, which may point to specificities that are not related to the hemicellulose.

Biochemical and Biophysical Properties of BoAgu115A—To explore the catalytic properties of BoAgu115A in more detail the activity of the enzyme against glucurono-xylooligosaccharides and pre-treated glucuronoxylan was evaluated using PACE. When Arabidopsis glucuronoxylan was treated with the GH30 glucuronoxylan-specific xylanase, BACOVA_03432, a range of products were observed with the most prominent species having a degree of polymerization of 7 to 11. The GH30 enzyme was inactive against the hemicellulose that had been pre-treated with BoAgu115A (Fig. 2C), indicating that glucuronidase can remove [Me]GlcA from all locations within the hemicellulose.

Glucurono-xylooligosaccharides were generated by digesting Arabidopsis glucuronoxylan to completion (i.e. the product profile remains stable) with GH10 or GH11 xylanases, which generated UXX and XUXX as the limit products, respectively (35). The data showed that BoAgu115A hydrolyzed UXX and XUXX (Fig. 3A), indicating that the enzyme cleaves GlcA from both non-reducing terminal and internal xylose residues, consistent with its activity against xylan, whereas the GH67 \( \alpha \)-glucuronidase CjGlcA67A released [Me]GlcA from UXX, but not from XUXX (data not shown), consistent with its capacity to remove the uronic acid only when it decorates the non-reducing terminal xylose (9, 11). When BoAgu115A was assayed against a mixture of terminally substituted aldouronic acids the activity of the enzyme followed the order UXXX > UXX > UX (Fig. 3B), demonstrat-
ing that the enzyme contains at least two positive subsites downstream of the +1 subsite (+2R and +3R). Positive subsites bind to the xylan backbone with the +1 subsite housing the xylose linked to the cleaved GlcA, and subsites that bind successively to sugars toward the reducing end of the xylan chain are labeled +R2, +R3 etc., whereas the subsites that bind to xylose toward the non-reducing end are defined as +NR2, +NR3 etc. (nomenclature described in Ref. 36). It was also evident that BoAgu115A was significantly more active against XUXX than UXX (Table 2), indicating that the glucuronidase contains at least one subsite upstream of the +1 subsite (+2NR). Against glucuronoxylans from birch and beech the $K_m$ of BoAgu115A is ~10-fold lower than XUXX, whereas the $k_{cat}$ of the enzyme is ~5-fold less against the polysaccharide compared with the oligosaccharide. Thus, although the $\alpha$-glucuronidase has a higher catalytic efficiency against xylan than XUXX, its lower $k_{cat}$ likely reflects tighter binding of the deglucuronylated xylan chains (i.e. reaction product) to the positive subsites, compared with the xyooligosaccharides released from the glucuronoxyloligosaccharides, resulting in slow product release and hence turnover rate. Thus, it is possible that the xylan binding site of BoAgu115A may be able to bind more than four xylose residues.

The pH optimum of BoAgu115A was ~7.0 (Fig. 4A) and when subjected to analytical ultracentrifugation migrated with a sedimentation coefficient of 8.78 ± 0.03 S, which equates to a molecular mass of 199 ± 6.7 kDa (Fig. 4B). Given that the 824-amino acid recombinant form of BoAgu115A has a molecular mass of 85 kDa, these data indicate that the glucuronidase is a dimer in solution, consistent with its crystal structure (see below).

Structure of BoAgu115A—The crystal structure of the native BoAgu115A was solved by extending the phase information from an initial selenomethionine SAD at 3.0 Å to a native resolution of 2.65 Å, whereas the ligand structure of the enzyme was determined by molecular replacement at a resolution of 2.14 Å (Table 1). The final model of BoAgu115A consists of one dimeric molecule in the asymmetric unit, with each protomer consisting of residues 33–856, with an average $B$ factor of 31.5 (Table 1). The final crystallographic $R$ value is 20.34, with an $R_{free}$ of 26.59 for the native apo model, 17.40 and 21.91, respectively, for the ligand bound model. BoAgu115A is an $\alpha/\beta$ globular protein with overall dimensions of about 100 × 70 × 50 Å per protomer. The enzyme consists of four distinct domains, which are connected by extended loops (Fig. 5A). The N-terminal domain, residues 33–196 (all residues are identified by their position in the full-length protein), comprises six $\beta$-strands that lay on top of two parallel $\alpha$-helices. The second domain (amino acids 197–482) displays a ($\beta/\alpha$)$_{6}$-fold (TIM barrel). This domain deviates slightly from a canonical TIM barrel; there is an additional helix between $\beta$-strands $\beta$-1 and $\beta$-2, and between $\beta$-3 and $\beta$-4, and the helices protruding from $\beta$-5 and $\beta$-6 are unusually short. The two $\alpha$-helices of the N-terminal domain make extensive contacts with the $\alpha$-helices extending from $\beta$-1 and $\beta$-2 of the TIM-barrel of the second domain. The third domain, extending from residues 488–641, comprises a five-

### TABLE 2

| Substrate          | $k_{cat}$ $\text{min}^{-1}$ | $k_m$ $\text{ms}^{-1}$ | $k_{cat}/k_m$ $\text{min}^{-1} \text{ms}^{-1}$ |
|--------------------|-----------------------------|-------------------------|-----------------------------------------------|
| XUXX               | 3233 (±187)                 | 4.5 (±0.54)             | 718                                           |
| UXX                | 1106 (±71)                  | 19.5 (±1.90)            | 57                                            |
| Beech wood xylan   | 696 (±38)                   | 0.4 (±0.06)             | 1740                                          |
| Birch wood xylan   | 665 (±24)                   | 0.3 (±0.04)             | 2217                                          |
| Beech wood xylan/GH30$^{a}$ | 837 (±77)             | 0.9 (±0.15)             | 930                                           |
| Birch wood xylan/GH30$^{a}$ | 862 (±64)             | 1.1 (±0.15)             | 784                                           |

$^{a}$ The polysaccharide was pre-digested to completion with a GH30 glucuronoxylan-specific xylanase.

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helical bundle. The C-terminal domain, amino acids residues 673–844, displays a canonical β-sheet/sandwich-fold consisting of two β-sheets each containing five anti-parallel β-strands in the following order: β-sheet 1 (concave surface), β-1, β-8, β-3, and β-6; β-sheet 2 (convex surface), β-2, β-7, β-4, and β-5. In general the β-strands are connected by loops, however, the loop
connecting β-1 and β-2 contains a pair of anti-parallel β-strands, whereas there is a short α-helix in the loop connecting β-5 with β-6. The two protomers in BoAgu115A display a “butterfly” like structure in which the two subunits make several interactions through the helical bundle domains. In addition, the C-terminal β-sandwich domain of protomer-1 makes extensive interactions with the TIM-barrel domain of protomer-2 and vice versa (Fig. 5, B and C).

Identifying the Active Site of BoAgu115A—Inspection of the surface of the BoAgu115A protomers revealed a cleft-like structure in the (β/α)8 domain that extends over the central β-barrel, on the opposite surface to the N-terminal domain. The center of the cleft, which may accommodate the xylan backbone, abuts onto a deep pocket that likely comprises the active site. This hypothesis was confirmed when BoAgu115A was co-crystallized with GlcA, which revealed electron density for the uronic acid in the proposed active site pocket (Figs. 5, B and C, 6, and 7). The sugar was found to be in its ring open conformation, which is highly unusual for hexaose aldehyde sugars. It is possible that the specific interactions between the uronic acid and the enzyme encourage GlcA to adopt an open chain conformation after glycosidic bond cleavage. The direct interactions between GlcA and the enzyme are as follows (Fig. 6): O3 makes a polar contact with Ne-1 of Trp-249. O6A and O6B of the uronic acid carboxylate form polar contacts with the OH of Tyr-420 and Ne-2 of His-422, respectively, the endocyclic oxygen interacts with the N of Val-426, and O1 makes a hydrogen bond with Asp-206. O4 is pointing into a hydrophobic open channel, comprising the side chains of Phe-203, Met-248, and Trp-245, which likely make apolar contacts with the methyl group of MeGlcA. Unlike the vast majority of glycoside hydrolases, the active site of BoAgu115A does not make typical parallel stacking interactions between the planar faces of aromatic residues and the sugar ring, although edge on edge apolar interactions between the sugar and two aromatic residues, Trp-420 and Tyr-420, and the aliphatic side chain of Val-426 were evident. The Oδ-2 of Asp-332 in the apo structure (the amino acid is not visible in the structure of the BoAgu115A-GlcA complex, discussed in detail below), is in an appropriate position to make polar contacts with O2 of GlcA, whereas Oδ-1 of Asp-206 forms a polar contact with O1 and may therefore act as the catalytic acid (Fig. 6).

To explore further the significance of the interactions between GlcA and BoAgu115A, and the function of other resi-
dues in the active site of the enzyme, targeted amino acids were substituted with alanine and the activity of the enzyme variants determined. The data, presented in Table 3 and Fig. 6, showed that mutating the residues that interact with the carboxylate of GlcA, His-422 and Tyr-420, resulted in a 105- and 103-fold reduction in activity, respectively, indicating that this component of the substrate is an important specificity determinant. O3 appears to interact with Trp-249 as the W249A mutation caused a 3000-fold reduction in catalytic activity. In addition to the residues that interact directly with the bound GlcA, several other amino acids in, or within the vicinity of, the active site were shown to make a significant contribution to the activity of the enzyme; the putative function of these residues is discussed below. Substituting amino acids near the putative xylan binding cleft/active site generally caused a modest reduction (5–100-fold) in the activity of the glucuronidase (see below), and mutating acid residues located in the other pocket/cleft-like structure on the enzyme (E162A, W169A, D192A, and D478A) did not affect activity (Table 3). These data are consistent with the view that the pocket located in the cleft-like structure in the TIM-barrel domain comprises the active site of BoAgu115A.

**FIGURE 7. The topology of the xylan binding cleft of BoAgu115A.** A and B show a solvent-exposed surface representation of the substrate binding cleft of protomer 1 of the apo (A) and GlcA bound (B) forms of BoAgu115A. In both panels the left-hand structures show a side on view down the putative xylan binding cleft (indicated with an arrow), and the right-hand structures provide a bird's eye view of the xylan binding cleft. In A and B, protomer 1 is colored light blue, protomer 2 is green, and the proximal wall (Trp-249) of the active site pocket is colored red. The flexible loop extending from Met-327 to Asn-346 is shown in cyan in B but not in A (the loop was too disordered to be modeled in the BoAgu115A-GlcA complex). C shows the position of the amino acids that interact (Lys-374, Glu-375) with residues in the flexible loop (Asp-328, Asp-332) in stick format with the carbons (and schematic of the overall fold) colored cyan (apo BoAgu115A) or green (BoAgu115A-GlcA complex), the carbons of GlcA are colored salmon pink, and all oxygens and nitrogens colored red and blue, respectively. Note the side chains of Lys-374, Glu-375, and Glu-782 are too disordered in the apo structure to be modeled. The polar contacts between the amino acids are shown by dotted lines and the distance in Å of these interactions are indicated.
ever, that the distance between Asp-332 and Glu-375 (in the apo structure) and the O1 of GlcA (in the structure of BoAgu115A in complex with GlcA) is 3.5 Å, respectively, are greater than the normal ~3.5 Å between the catalytic base and the anomeric carbon of the glucose sugar in inverting glycoside hydrolases (Fig. 6). It is possible that BoAgu115A displays a "Grotthus"-style mechanism in which a remote amino acid activates the active site nucleophilic water via a string of solvent molecules (37), as proposed for some inverting glycoside hydrolases (37, 38). In such a mechanism the distance between the catalytic base and the sugar is not restricted to 3.5 Å. Both Asp-332 and Glu-375, however, are on highly mobile loops that have high B-factors. Indeed, in the BoAgu115A-GlcA structure the loop that contains Asp-332 (Met-327 to Asn-346) was too disordered to be modeled, whereas Glu-375 could not be built past the β-carbon (Fig. 6). Thus, as discussed below, in the Michaelis catalytic complex Glu-375 and Asp-332 may adopt conformations, not observed in the apo and product structures, which reveal their true function in substrate binding and catalysis.

In enzymes and non-catalytic binding proteins that interact with glucuronic acid, arginines are often important specificity determinates, making bidentate hydrogen bonds/salt bridges with the carboxylate group (9, 39). It is possible that in BoAgu115A Arg-328 contributes to substrate binding by also interacting with the carboxylate of the glucuronic acid substrate, a view supported by the observation that the R328A mutation caused ~10^6-fold decrease in activity (Table 3). Arg-328 (and Glu-375) in the apo structure, however, are too distant from the hub of the active site to play a direct role in enzyme function (Fig. 6). Thus, for the arginine to contribute to substrate binding the loop containing this amino acid (and Asp-332) would be required to undergo a conformational change. Another possibility is that the role of Arg-328 is to stabilize the conformation of Asp-332, which may function as the catalytic base, whereas the primary role of Glu-375 could be to orientate the guanidino group of the arginine toward the aspartate. To conclude, the above structural and mutagenesis data indicate that the assembly of the Michaelis complex in BoAgu115A requires the repositioning of several flexible elements, most notably the loop carrying Asp-332 and Arg-328. Indeed, close inspection of the apo structure reveals that the conformation of this loop, which forms a component of the xylan binding cleft, in addition to part of the active site pocket, likely plays a central role in both glucuronoxylan binding and departure of the reaction products, both GlcA and the undecorated xylose polymer (Fig. 7, A and B). Furthermore, the loop also plays a role in stabilizing residues in the putative xylan binding cleft, notably Lys-374 and Glu-782, whose side chains are disordered in the GlcA bound structure where the loop is too flexible to be modeled (Fig. 7C). Although conformational changes are unusual in glycoside hydrolases, they are not without precedent, exemplified by the substantial movement of the catalytic TIM-barrel domain induced by substrate binding observed in GH112 glycoside phosphorolases (40).

### TABLE 3

| Activity of mutants of BoAgu115A | Catalytic activity^a |
|----------------------------------|----------------------|
| BoAgu115A derivative             | αcat/Κm min⁻¹ μmol⁻¹ |
| Wild type                        | 780.0                |
| E162A                            | ~780.0^b             |
| D192A                            | ~780.0^b             |
| W169A                            | ~780.0^b             |
| D478A                            | ~780.0^b             |
| E785A                            | 198.0                |
| Y788A                            | 152.0                |
| E782A                            | 123.0                |
| N205A                            | 44.0                 |
| Y425A                            | 10.8                 |
| Y324A                            | 8.0                  |
| Y792A                            | 7.6                  |
| D396N                            | 5.4                  |
| Y373A                            | 3.7                  |
| D206A                            | 2.5                  |
| N462A                            | 1.8                  |
| Y373A                            | 1.1                  |
| Y420A                            | 0.8                  |
| H275A                            | 0.3                  |
| W249A                            | 0.28                 |
| E375A                            | 0.17                 |
| H422A                            | 0.01                 |
| R328A                            | 0.01                 |
| H275A/H422A                      | ND^c                 |
| D332A                            | ND                   |
| ΔC-terminal (1–526)              | ND                   |
| ΔC-terminal (1–639)              | ND                   |
| ΔC-terminal (1–665)              | ND                   |

^a Wild type and mutants of BoAgu115A were assayed using 1 mM XUXX as the substrate in 20 mM sodium phosphate buffer, pH 7.0, containing 1 mg ml⁻¹ of BSA. At this substrate concentration the initial rate provides a direct readout of the catalytic efficiency of the enzyme.

^b ~780; the activity of the mutants of residues from the other cleft/pocket-like structure on the enzyme was estimated from a single time point reaction.

^c ND, no activity could be determined using an assay that can detect activity that is ~10⁻fold less than the wild type enzyme.

The Possible Assembly of the Catalytic Apparatus of BoAgu115A—GH115 enzymes are inverting glycoside hydrolase (14) and thus glycosidic bond cleavage is catalyzed by an acid base-assisted single displacement mechanism. In such a mechanism the catalytic Brønsted base, typically a carboxylate, activates a water molecule that attacks the anomeric carbon of the glycone sugar, whereas the catalytic acid, also a carboxylic acid residue, donates a proton to the glycosidic oxygen promoting leaving group departure (14). As stated above, Asp-206, based on its interaction with O1 of GlcA, may comprise the catalytic acid. Support for this view is provided by the apolar environment of the aspartate; the carboxylate amino acid is in close proximity to Trp-249 and Val-426, promoting an elevated pKₐ. Furthermore, O6-2 of Asp-206 is within hydrogen bonding distance with O6-1 of Asp-424, and thus may function as the pKₐ modulator of the putative catalytic acid (Fig. 6). It should be emphasized, however, that the assignment of Asp-206 as the catalytic residue must be viewed with some caution as the D206A substitution resulted in only a 300-fold reduction in activity, and, as the bound GlcA is in an open chain form, the position of O1 may not reflect the location of the atom when the uronic acid is in a closed pyranose configuration, adopted in the substrate.

Based on mutagenesis data the other carboxylic acid amino acids in the vicinity of the active site, Asp-332 and Glu-375, are candidate catalytic residues; the D332A and E375A mutations caused complete inactivation of the enzyme and a ~5000-fold reduction in activity, respectively (Table 3). It is evident, however, that the distance between Asp-332 and Glu-375 (in the apo structure) and the O1 of GlcA (in the structure of BoAgu115A in complex with GlcA) is 3.5 Å, and, respectively, are greater than the normal ~3.5 Å between the catalytic base and the anomeric carbon of the glucose sugar in inverting glycoside hydrolases (Fig. 6). It is possible that BoAgu115A displays a "Grotthus"-style mechanism in which a remote amino acid activates the active site nucleophilic water via a string of solvent molecules (37), as proposed for some inverting glycoside hydrolases (37, 38). In such a mechanism the distance between the catalytic base and the sugar is not restricted to 3.5 Å. Both Asp-332 and Glu-375, however, are on highly mobile loops that have high B-factors. Indeed, in the BoAgu115A-GlcA structure the loop that contains Asp-332 (Met-327 to Asn-346) was too disordered to be modeled, whereas Glu-375 could not be built past the β-carbon (Fig. 6). Thus, as discussed below, in the Michaelis catalytic complex Glu-375 and Asp-332 may adopt conformations, not observed in the apo and product structures, which reveal their true function in substrate binding and catalysis.

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Whether Asp-332, or possibly Glu-375, comprise the actual catalytic base is currently unclear, as is the role of Arg-328 in substrate binding, as a Michaelis complex could not be...
obtained. Although the catalytic apparatus of glycoside hydrolases generally comprise two carbohydrate residues, exceptions are evident with histidine acting as a catalytic acid-base in a GH3 N-acetylglucosaminidase (41), and as a catalytic acid in an inverting GH117 anhydro-L-galactosidase (42). Given that alanine substitutions were made of Glu-782, Glu-785, Tyr-788, and Tyr-792, whose side chains line the proposed xylan binding cleft. The 100-fold reduction in catalytic activity mediated by the Y792A substitution (Table 3) indicated that Tyr-792 makes a significant contribution to xylan binding, whereas removal of the C-terminal domain (enzyme truncated at residue Ile-665) resulted in the complete abrogation of enzyme activity, supporting a key role for this structural element in the topology of the catalytic apparatus.

The Potential Role of the C-terminal Domain of BoAgu115A—Inspection of the dimeric form of BoAgu115A (Fig. 9A) suggests that the C-terminal β-sandwich domain of protomer 2 contributes to one of the faces of the likely xylan binding cleft of protomer 1 and vice versa (Fig. 7). To explore the role of the C-terminal domain in the activity of the enzyme, alanine substitutions were made of Glu-782, Glu-785, Tyr-788, and Tyr-792, whose side chains line the proposed xylan binding cleft. The 100-fold reduction in catalytic activity mediated by the Y792A substitution (Table 3) indicated that Tyr-792 makes a significant contribution to xylan binding, whereas removal of the C-terminal domain (enzyme truncated at residue Ile-665) resulted in the complete abrogation of enzyme activity (Table 3), supporting a key role for this structural element in the topology of the GH115 catalytic apparatus.

Structural Comparison of GH115 and GH67 α-Glucuronidases—Structural comparison of BoAgu115A with the PDB database (using DaliLite version 3) identified the Geobacillus stearothermophilus GH67 α-glucuronidase, AgaA (11), as the closest, albeit weak, structural homolog with a Z-score of 16.1, root mean square deviation of 4.7 Å, and sequence identity of 11% over 470 aligned residues. Despite the weak structural homology and sequence similarity, the first three domains of the two proteins display the same fold and, intriguingly, the location of the active site of the GH115 and GH67 enzymes, endo- and exo-acting xylan-specific α-glucuronidase, respectively, is conserved, pointing to a distant evolutionary link between the two families (Fig. 9A). It is evident, however, that there is no conservation of the active site residues, although it should be noted that the catalytic acid in GH67 enzymes is positioned on a highly mobile loop that can only be stabilized in the presence of ligands (11), which has some resonance with the mobile loop containing the catalytically significant residues Arg-328 and Asp-332 in BoAgu115A (Fig. 9B). In addition, the topology of the active site of the two enzymes is very different, comprising a deep pocket embedded in a rigid blind canyon or a tunnel (reviewed by Refs. 5 and 6). It is intriguing, therefore, that despite significant structural conservation, indicating an evolutionary link between the two glucuronidases families, the region of the enzyme that are most likely to be highly conserved, the catalytic apparatus of glycoside hydrolases generally comprise two carbohydrate residues, exceptions are evident with histidine acting as a catalytic acid-base in a GH3 N-acetylglucosaminidase (41), and as a catalytic acid in an inverting GH117 anhydro-L-galactosidase (42). Given that alanine substitutions were made of Glu-782, Glu-785, Tyr-788, and Tyr-792, whose side chains line the proposed xylan binding cleft. The 100-fold reduction in catalytic activity mediated by the Y792A substitution (Table 3) indicated that Tyr-792 makes a significant contribution to xylan binding, whereas removal of the C-terminal domain (enzyme truncated at residue Ile-665) resulted in the complete abrogation of enzyme activity (Table 3), supporting a key role for this structural element in the topology of the GH115 catalytic apparatus.

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lytic apparatus, is clearly different in GH67 and GH115. The evolutionary selection pressures that led to such diversity in the active site of structurally and functionally related enzymes families are currently unclear.

Conclusions—This report reveals the first structure of a GH115 enzyme, identifying a likely distant evolutionary link to GH67, the other major family of α-glucuronidases. The locations of the residues that contribute to activity indicate that the enzyme undergoes a substantial conformational change to assemble a functional catalytic apparatus. It is interesting to note that despite complete conservation of the catalytically important amino acids in seven GH115 proteins, only BoAgu115A was shown to display activity (against glucuronoxylan). Although it is possible that the other six Bo ovatus GH115 proteins are not catalytically active, a more likely explanation is that their target uronic acid-containing substrates are not glucuronoxylan, suggesting that GH115 is a poly-specific family.

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