Structural and biochemical characterization of glycoside hydrolase family 79 β-glucuronidase from *Acidobacterium capsulatum**

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SUPPLEMENTAL MATERIAL

Experimental Procedures

Substrates.

p-Nitrophenyl-glycosides (PNP-glycosides) (such as PNP-α-L-arabinopyranoside, PNP-β-L-arabinopyranoside, PNP-α-L-arabinofuranoside, PNP-α-galactopyranoside, PNP-β-galactopyranoside, PNP-α-glucopyranoside, PNP-β-glucopyranoside (PNP-β-Glc), PNP-α-mannopyranoside, PNP-β-mannopyranoside, PNP-α-xylopyranoside, PNP-β-xylopyranoside (PNP-β-Xyl), PNP-α-L-fucopyranoside, PNP-β-fucopyranoside, PNP-α-L-rhamnopyranoside, PNP-α-galacturonide, PNP-β-glucuronide (PNP-β-GlcA), PNP-α-*N*-acetylgalactosaminide, PNP-β-*N*-acetylgalactosaminide, PNP-α-*N*-acetylglucosaminide, PNP-β-*N*-acetylglucosaminide, PNP-β-lactopyranoside and PNP-β-cellobioside) were purchased from Sigma. 4-*O*-Methyl-GlcA-β-1,6-Gal-β-1,6-Gal (MeGlcA-β-1,6-Gal_2) was prepared as previously (Tsumuraya et al., 1990).

Crystallization, Data Collection, and Structural Determination.

Initial crystallization screening of AcGlcA79A was conducted by the sitting-drop, vapor-diffusion method at 20°C, mixing 0.3 μl of the protein solution (4.6 mg ml^{-1}) and an equal volume of precipitant against 50 μl of reservoir solution using the following commercially available kits: JCSG+ Suite (Qiagen, Düsseldorf, Germany), Crystal Screen HT, and Index HT (Hampton Research, Aliso Viejo, CA, USA). Among 3 × 96 trials, solution number 18 of Index HT (1.4 M sodium/potassium phosphate, pH 6.9) gave small pieces of rod-shaped crystals. After refinement of the crystallization conditions, AcGlcA79A was crystallized by the sitting-drop, vapor-diffusion method with the precipitant solution composed of 2.0 M sodium phosphate monobasic monohydrate/potassium phosphate dibasic (0.5/9.5 [v/v], pH not adjusted) and with a protein concentration of 2.5 mg ml^{-1}. Crystals with maximum dimensions of 200 × 200 × 500 μm were consistently obtained within a few days at 20°C. Selenomethionine (Se-Met)-labeled AcGlcA79A was produced using the *E. coli* B834 (DE3) methionine auxotroph and was crystallized under the same conditions as for the native enzyme. The GlcA complex was prepared by soaking the AcGlcA79A crystals in crystallization liquor containing GlcA powder for five minutes. The fluorinated glucuronide (2FGlcA) intermediate complex was prepared by adding the crystallization liquor containing 0.3% (w/v) DNP-2FGlcA into the AcGlcA79A crystallization drop and incubating for five minutes.

Diffraction experiments were conducted at the Photon Factory (PF) or the PF-Advanced Ring (PF-AR), High Energy Accelerator Research Organization, Tsukuba, Japan. The crystals were moved into the mother liquor containing 20% (v/v) glycerol as a cryoprotectant, and a single crystal was scooped in a nylon loop and flash-frozen in a nitrogen gas stream at -178°C. Diffraction data were collected with the Quantum 270 CCD detector (ADSC, Poway, CA, USA). Native data were collected with 1-s exposures for 0.5° oscillations for a total of 180° at a wavelength of 1.00 Å. Se-Met peak data were collected with 2-s exposures for 1° oscillations for a total of 360°, and edge and high remote data were collected for a total of 90°. GlcA complex data were collected with 1-s exposures for 0.5° oscillations for a total of 180°. 2FGlcA complex data were collected with 2-s exposures for 1° oscillations for a total of 360°. Data were integrated and scaled using the programs *DENZO* and *Scalepack* in the *HKL2000* program suite (Otwinowski and Minor, 1997). AcGlcA79A crystals diffracted to approximately 1.4 Å resolution and belonged to the space group $I4_122$. Assuming that the asymmetric unit of the crystal contained one AcGlcA79A molecule, the Matthews coefficient was calculated to be 2.7 $Å^3$ Da^{-1} (Matthews, 1968); this corresponded to the 55% solvent content of the crystals.

Structural analysis of AcGlcA79A was conducted through the multiwavelength anomalous dispersion method using Se-Met-labeled AcGlcA79A crystals. Seven selenium atom positions were determined, and initial phases were calculated using the AutoSol and AutoBuild wizards in the PHENIX program suite (Adams et al. 2010). The obtained initial model was further improved using the program ARP/Warp (Cohen et al., 2004). Manual model rebuilding, introduction of water molecules, and molecular refinement were conducted using Coot and Refmac5 (Emsley and Cowtan, 2004; Murshudov et al., 1997). One phosphate ion and several glycerol molecules were added into the model. For the analyses of GlcA- and 2FGlcA-binding structures of AcGlcA79A, structural determination was conducted using the ligand-free structure as the starting model, and the bound ligand was observed in the difference electron

density map. Refinement parameters for the ligands were generated by the GlycoBioChem PRODRG2 Server (Schüttelkopf and van Aalten, 2004). Data collection and structure refinement statistics are given in Table 1. Stereochemistry of the models was analyzed with the program Rampage (Lovell et al., 2003) in the CCP4 program suite (Bailey, 1994). Structural drawings were prepared by the program PyMol (DeLano Scientific LLC, Palo Alto, CA, USA).

Expression of AcGlcA79A and Mutant Generation.

Acidobacterium capsulatum NBRC15755 was obtained from the National Institute of Technology and Evaluation (Kazusa, Japan). Genomic DNA from *A. capsulatum* was prepared by using InstaGene Matrix (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's instructions. The gene encoding a putative β-glucuronidase (ACP_2665; GenBank accession number AC032043) was amplified from *A. capsulatum* genomic DNA by PCR using Phusion DNA polymerase (Finnzymes, Espoo, Finland) and the following primers: forward, 5′-CAT ATG GCT TTT GCC CGC GGC GGA CTG GCT -3′; reverse, 5′-AAG CTT AGC GAA TTC GAG CAA TGC GCC GGA-3′. The amplified DNA was cloned into pET30(+) (Novagen, Darmstadt, Germany) at NdeI and HindIII restriction enzyme sites (underlined). *Escherichia coli* BL21gold (DE3) (Novagen) cells harboring the expression plasmid were cultured in Luria-Bertani medium containing 25 μg/ml kanamycin at 37°C to an A_{600} of 0.6, and expression was induced with 1 mM isopropyl-β-thiogalactopyranoside for additional 24 h at 25°C. The recombinant enzyme (AcGlcA79A) was purified with Ni nitrilo-triacetic acid-agarose (Qiagen GmbH, Hilden, Germany). The enzyme was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The relevant fractions were pooled and dialyzed against deionized water. The final preparation thus obtained was used as purified enzyme. The enzyme concentration was determined by measuring A_{280}, assuming that an absorbance of 1.0 indicated a concentration of 1 mg/ml. Amino acid substitutions of AcGlcA79A were generated by inverse PCR using pET30/AcGlcA79A as template DNA and the appropriate primers. The PCR product was purified, ligated, and transformed into *E. coli* DH5α. Mutations were confirmed by DNA sequencing. Expression and purification of mutants were carried out in the same way as for wild type AcGlcA79A.

Enzyme Assays.

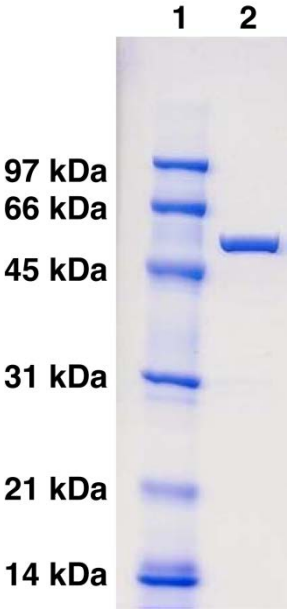
β-Glucuronidase activity was determined using a mixture containing 25 μl of 2 mM PNP-β-GlcA, 20 μl of McIlvaine buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid, pH 3.0), and 5 μl of enzyme solution. The reactions were carried out at 37 °C for 10 min and terminated by the addition of 50 μl of 0.2 M Na_2CO_3. The amount of PNP released was detected at A_{400} (extinction coefficient = 7284 M^{-1}•cm^{-1}). One unit of enzyme activity is defined as the amount of enzyme that released 1 μmol of PNP per minute. The effects of pH and temperature on enzyme activity were investigated as described previously (Ichinose et al. 2008). The following buffers were used to study the effect of pH on β-glucuronidase activity: glycine-HCl buffer (pH 2.0–3.5), McIlvaine buffer (pH 2.6–7.6), acetate buffer (pH 3.5–6.0) and Atkins-Pantin buffer (pH 7.6–11.0). The activity of β-glucuronidase was assayed under the conditions described above. The effect of pH on enzyme activity was assayed with or without 90 mM formic acid or 90 mM acetic acid in McIlvaine buffer as described above.

The substrate specificity of AcGlcA79A toward various PNP-glycosides was determined. The assay method was identical to that described for PNP-β-GlcA. The kinetic parameters of wild-type and mutants of AcGlcA79A against PNP-β-GlcA, PNP-β-Glc and PNP-β-Xyl were determined as follows. The reactions were performed in McIlvaine buffer (pH 3.5) containing 0.01–10 mM substrates, 0.1% (w/v) bovine serum albumin, and 0.9 nM–10.0 mM enzyme at 37 °C for up to 10 min. The amount of PNP released was determined from the A_{400}. The kinetic parameters k_{cat} and K_m were determined by the Lineweaver-Burk plot from three independent experiments, and at five substrate concentrations. The substrate specificity and the catalytic efficiency of wild type and mutants of AcGlcA79A were analyzed using MeGlcA-β-1,6-Gal_2. Briefly, the enzyme (5 μM) was incubated with substrate (10 μM) in McIlvaine buffer, pH 3.5, at 37°C. At regular time intervals, the amount of degradation of each substrate was quantified by high performance anion-exchange chromatography with pulsed amperometric detection (Ichinose et al., 2005). The assay was performed in dupulicate.

Determination of stereochemistry of hydrolysis by ^{1}H-NMR.

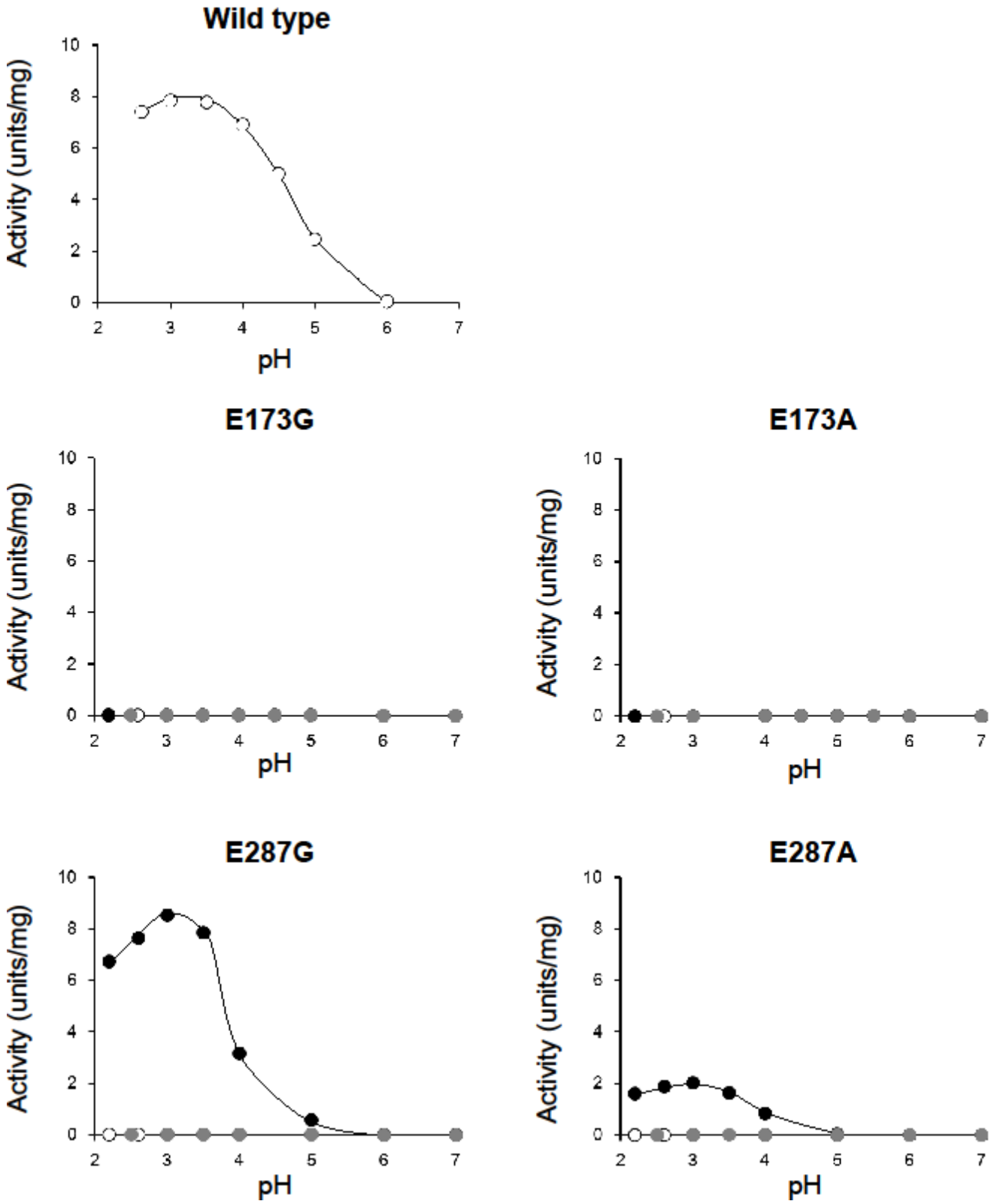
Substrate and recombinant enzyme were lyophilized twice from D_2O before use. A solution of 15 mM PNP-β-GlcA (Koch-Light, England) in 0.04 M deuterized sodium acetate buffer (pD 3.7) was incubated with 0.5 mg of AcGlcA79A. This enzyme concentration was needed to achieve a rapid substrate hydrolysis to avoid the effect of mutarotation on determination of the anomeric configuration of the primary product of hydrolysis. ^{1}H-NMR spectra were recorded at different time intervals on a Varian 400-MR (400 MHz) spectrometer.

Fig. S1.
SDS-PAGE analysis of recombinant AcGlcA79A.



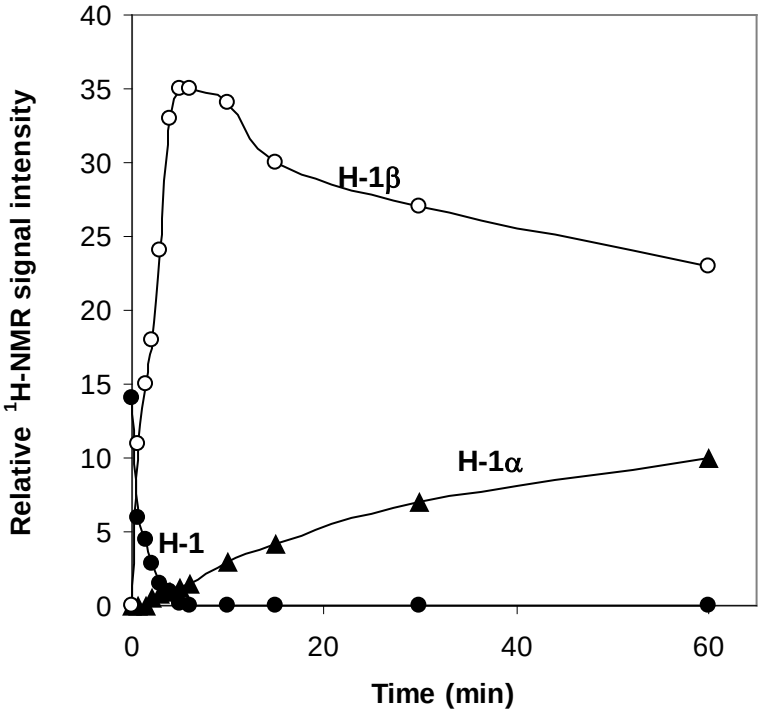
Lane 1, molecular mass marker (1 μg each band); lane 2, purified AcGlcA79A (1 μg).

Fig. S2.
pH-activity profiles of AcGlcA79A wild type and the mutants with or without chemicals.



Shown is an activity versus pH in McIlvaine buffer alone (open circle), with 90 mM formic acid (black circle) or with 90 mM acetate acid (gray circle).

Fig. S3.
Time course of hydrolysis of PNP-β-GlcA with AcGlcA79A followed by ^{1}H-NMR spectroscopy.



The relative intensities of H-1 signals of the substrate and product are plotted versus time.