Structural basis for cyclophellitol inhibition of a β-glucosidase

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The structural basis for β-glucosidase inhibition by cyclophellitol is demonstrated using X-ray crystallography, enzyme kinetics and mass spectrometry.

Glycosidase inhibition is extremely attractive in the quest for therapeutic agents for the treatment of diseases such as diabetes, viral infections and cancer. The study of enzymes in complex with inhibitors aids development of inhibitor design and also the understanding of the glycosidase mechanism.[1] The 'trapping' of mechanism-based inhibitors to the catalytic residues of enzymes has greatly advanced our knowledge of glycosidase hydrolysis.[2]

Glycoside hydrolysis with retention of anomeric configuration proceeds with a two-step double displacement mechanism (Fig. 1). During the first step the acid/base residue protonates the glycosidic oxygen to aid leaving group departure, and the nucleophile attacks at the anomeric carbon to form a covalent glycosyl-enzyme intermediate. The glycosyl-enzyme ester is subsequently hydrolysed by a water molecule that has been activated by the acid/base residue. Compounds that covalently bind to the enzyme have helped to demonstrate unequivocally that the retaining mechanism for glycoside hydrolysis passes through a covalent intermediate, and have aided studies to identify catalytic residues.[3,4] This study examines the binding of cyclophellitol **1** to a β-glucosidase from

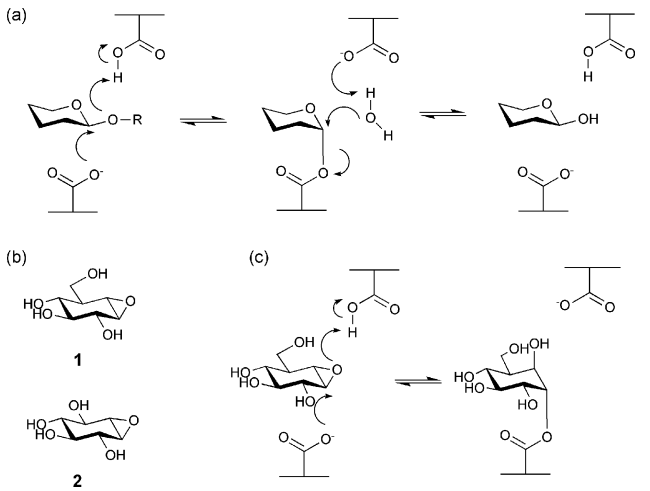

Fig. 1 (a) β-Glycoside hydrolysis with retention of anomeric configuration; (b) Cyclophellitol **1** and conduritol B epoxide **2**; (c) Proposed mechanism for covalent intermediate trapping by cyclophellitol.

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Thermotoga maritima (*Tm*GH1). Compound **1** was first isolated from a culture filtrate of a mushroom strain, *Phellinus* sp.,[5] and later synthesised chemically by a number of groups.[6–8] Others have reported the inhibition by **1**,[9–11] and demonstrated it can induce Gaucher's-like disease in mice, presumably by inhibiting β-glucocerebrosidase.[9] A mechanism has been proposed for its inhibitory activity, but to date there has been no structural evidence provided for this. Historically, more interest has been paid to conduritol B epoxide **2**,[12,13] which differs from **1** in that it possesses a hydroxyl group at C5 instead of a hydroxymethyl group. The intrinsic symmetry of **2** means that it can bind in two orientations and thus inhibit both α- and β-glucosidases. Cyclophellitol **1** is considered to be considerably more potent than **2**,[10,14] and also shows specificity for β-glucosidases only.

Pioneering work in the late 1980s by Withers and colleagues led to the use of 2-fluoro derived sugars as mechanism-based inhibitors for glycosidases.[15] More recently such fluoro sugars have notably been used in the elucidation of the mechanisms for hen egg white lysozyme[16] and sialidases,[17] where the existence of a covalent intermediate had been questioned. These compounds possess a good leaving group to ensure the first step of the mechanism is fast, but the electron-withdrawing halide at C2 means turnover of the glycosyl-enzyme intermediate is slow. An ever-increasing number of three-dimensional structures has been solved with 2-fluoro derived sugars bound to the enzyme to reveal the diverse sugar conformations adoped by different enzymes (some examples from the authors' own laboratory include ref. 18–20). Despite the wealth of structural information on 2-fluoro compounds, little structural work has been done on **1** or **2**. Compound **2** has been observed covalently bound to a plant β-D-glucan glucohydrolase[21] and the human acid β-glucosidase,[22] but this report provides the first structure of **1** in complex with an enzyme. Compounds **1** and **2** differ from the 2-fluoro compounds as they cause irreversible inactivation of the enzyme (the mechanism is depicted in Fig. 1). As with hydrolysis of glycosides, there will be nucleophilic attack at the anomeric carbon, which will be concomitant with protonation of the unstable epoxide oxygen. This will form an axial hydroxyl group at the C7 position (where an endocyclic oxygen is found in a native glycoside) and a covalent inhibitor-enzyme species. The absence of the endocyclic oxygen causes a highly stable covalent inhibitor-enzyme bond, which cannot be broken down by an activated water molecule.

*Tm*GH1 was co-crystallised in the presence of **1** and the structure subsequently solved by X-ray crystallography with data to 1.9 Å resolution[23] (Table 1). There is clear electron density in the active site corresponding to a molecule of **1** that had reacted with the enzyme to form a species in a $^4C_1$ chair conformation, with an axial hydroxyl group at C7, covalently linked *via* C1 to the nucleophile of *Tm*GH1, Glu351 (Fig. 2). Most of the interactions made by the cyclophellitol-derived compound with the active site

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Table 1 Data processing and refinement statistics

Resolution (outer shell) (Å)	30–1.90 (1.97–1.90)
Space group	$P2_12_12_1$
Unit cell parameters	$a = 94.5$ Å, $b = 94.7$ Å, $c = 113.6$ Å
R_{merge} (outer shell)	0.045 (0.279)
Mean $I/\sigma I$ (outer shell)	19.2 (3.5)
Completeness (outer shell) (%)	94.6 (78.3)
Multiplicity (outer shell)	3.3 (2.4)
No. unique reflections	76161
R_{cryst}	0.196
R_{free}	0.244
RMSD bonds (Å)	0.015
RMSD angles (°)	1.44
RMSD chiral volume (Å^3)	0.104
No. protein atoms	7226
No. ligand atoms	24
No. water atoms	476
No. other atoms	9
Average main chain *B*-factor (Å^2)	36
Average side chain *B*-factor (Å^2)	36
Average ligand *B*-factor (Å^2)	24
Average water *B*-factor (Å^2)	42
PDB code	2JAL

residues are similar to those described previously for *Tm*GH1.[20] The hydroxyl group at C6 hydrogen bonds with Glu405, the C4 hydroxyl group with Gln20 and Glu405 and the hydroxyl group at C3 with Gln20, Trp406 and His121. The hydroxyl group at C2 interacts with His121 and a water molecule. The additional hydroxyl group at C7, caused by the reaction of the epoxide, makes a hydrogen bond interaction with a water molecule. The formation of the covalent bond with the nucleophile, Glu351, causes rotation around the Cβ–Cγ bond, presumably to avoid a clash between O2 of the cyclophellitol-derived compound and Oε2 of Glu351. The observation of such rotation of the nucleophile is common with covalently bound inhibitors.[18,20] Tyr295, which interacts with the endocyclic oxygen in the 2-fluoro glucose complex with *Tm*GH1,[20] is seen to move ~90° to avoid unfavourable interactions with the C7 atom in the cyclophellitol-derived compound. The residue at this position is predicted to be important for transition state stabilisation, and if removed has a significant impact on the catalytic rate.[24]

In addition to the three-dimensional visualisation, electrospray mass spectrometry was used to observe the nature of the covalent complex.[26] Measurements revealed a m/z of 53 740 Da for native *Tm*GH1 and 53 917 Da for *Tm*GH1 with the addition of **1**; the difference of 177 Da corresponds to the addition of the reacted cyclophellitol compound, and thus demonstrates that the bond is truly covalent. Mass spectrometry determinations made ~96 hours after the addition of **1** to *Tm*GH1 show no decrease in mass and confirm the irreversible nature of the interaction.

Kinetic studies have demonstrated previously that **1** is a potent inhibitor of β-glucosidases, and indeed this was also seen to be the case with *Tm*GH1[27] (Fig. 3). Inhibition follows pseudo-first order kinetics of inactivation, as expected for an inhibitor that binds reversibly prior to covalent bond formation. Fitting of the pseudo-first order rate constants at each inhibitor concentration

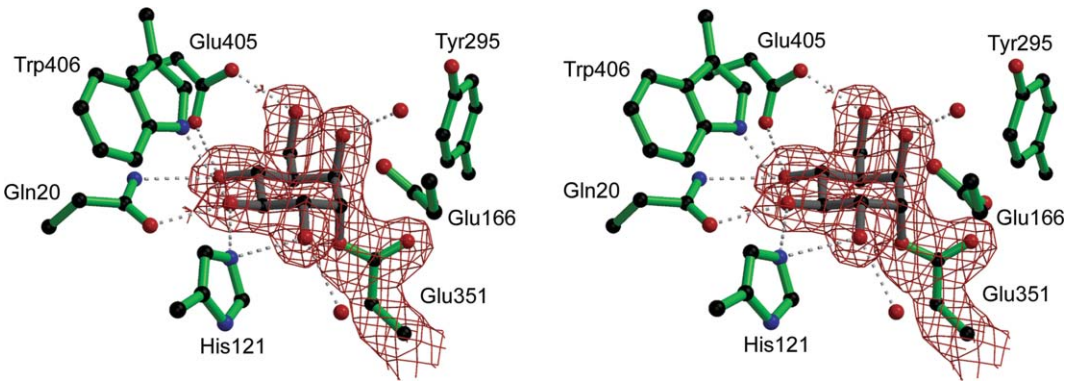

Fig. 2 Ball-and-stick representation of *Tm*GH1 with the enzyme reacted form of **1**, with interacting active site residues shown. Observed electron density for the maximum likelihood weighted $2F_{obs}–F_{calc}$ map is contoured at 1σ (~0.25 e Å^{-3}); the figure was drawn using BOBSCRIPT.[25]

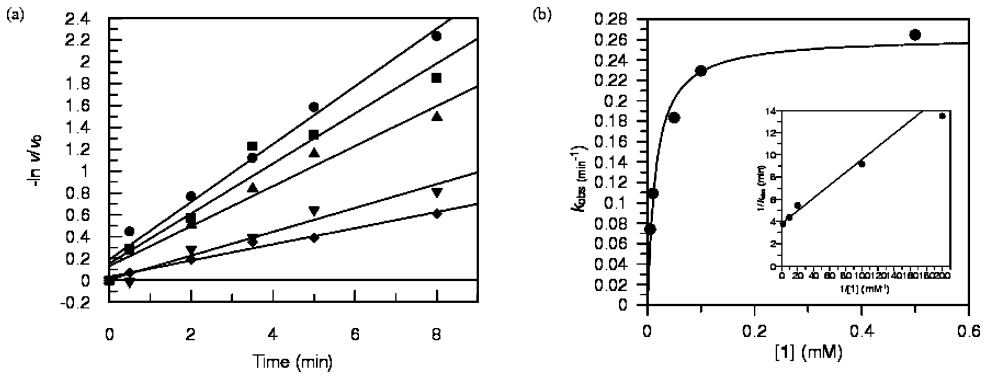

Fig. 3 (a) Semilogarithmic plots of residual activity of *Tm*GH1 *versus* time at 0.5 mM (circles), 0.1 mM (squares), 0.05 mM (triangles), 0.01 mM (inverted triangles) and 0.005 mM (diamonds) **1**; (b) Fitting to Equation (1) of pseudo-first order rate constants at different inhibitor concentrations.[27]

gives a K_i (equilibrium constant for initial binding) of 15 μM and a k_i (inactivation rate constant) of 0.26 min^{-1}. The K_i value is comparable to previous determinations with the almond β-glucosidase (2.8 μM[9] and 340 μM[10]), although the purity of commercially available preparations of those previous proteins remain questionable. The samples used for the *Tm*GH1 kinetics were tested for activity after extensive dialysis into buffer, but *Tm*GH1 remained totally inactive demonstrating that **1** binds irreversibly, as seen previously.[9,10]

This study provides the first structural basis for the inactivation of a β-glucosidase by cyclophellitol. As previous studies have suggested, a covalent bond is formed with the nucleophile, and this is irreversible. The inhibition is potent, with a K_i value for initial binding of a similar magnitude to many glycosidase inhibitors touted as transition-state mimics. In addition, the slow onset inhibition observed with many of these inhibitors, means that **1** acts on a similar time-scale to many glycosidase inhibitors, but binds irreversibly which may be advantageous for some applications. Previous studies have shown that **1** has excellent selectivity against β-glucosidases, rather than α-glucosidases,[9,10] and as such remains a versatile and selective probe for investigating enzyme mechanism *in vitro* and *in vivo.*

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23 *Tm*GH1 was crystallised as described previously [D. L. Zechel, A. B. Boraston, T. Gloster, C. M. Boraston, J. M. Macdonald, D. M. G. Tilbrook, R. V. Stick and G. J. Davies, *J. Am. Chem. Soc.*, 2003, **125**, 14313–14323] in the presence of 2 mM **1** and flash frozen. Data were collected on a beamline ID23-1 at the European Synchrotron Radiation Facility. All crystallographic computing was performed using the CCP4 suite of programs [Collaborative Computational Project Number 4, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 1994, **50**, 760–763].
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25 R. M. Esnouf, *J. Mol. Graphics Modell.*, 1997, **15**, 132–134.
26 Mass spectrometry studies were performed using a quadrupole time-of-flight electrospray source (Applied Biosystems Qstar) with *Tm*GH1 in the absence and presence of 100 mM **1**.
27 Inactivation kinetics studies were performed by monitoring UV–visible absorbance at 25 °C. *Tm*GH1 (at 0.37 μM) was incubated with between 5 μM and 0.5 mM **1**. At time intervals 10 μL of the enzyme–inhibitor mix were assayed for residual activity using 100 μM 2,4-dinitrophenyl β-D-glucopyranoside as substrate. 2,4-Dinitrophenolate release was monitored for 60 s at 400 nm to determine the rate. The log of the residual activity was plotted against time to obtain pseudo-first order rate constants. These were plotted against the inhibitor concentration and fitted to Equation (1) in GRAFIT [R. J. Leatherbarrow, *GraFit Version 5*, Erithacus Software Ltd., Horley, UK, 2001] to determine the dissociation constant K_i and maximal inactivation rate constant k_i [Equation (1): $k_{obs} = (k_i[I])/(K_i + [I])$].

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