Common Inhibition of Both β-Glucosidases and β-Mannosidases by Isofagomine Lactam Reflects Different Conformational Itineraries for Pyranoside Hydrolysis

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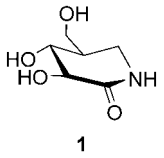
The ninety sequence-based families of glycoside hydrolases (GHs)[1] and the correspondingly large diversity of protein topologies[2] are a rich framework for studying variations in the catalytic mechanism of enzymatic glycoside hydrolysis. Such hydrolysis features oxocarbenium-ion-like transition states in which the anomeric centre becomes sp^2 hybridised and partial positive charge accumulates, primarily across the endocyclic O5–C1 bond. For pyranosides, such a species demands planarity of C5, O5, C1 and C2 at or near the transition state; a situation accommodated only by the $^4H_3$ and $^3H_4$ (half-chair) conformations (or their closely related envelope forms) and $^{2,5}B$ and $B_{2,5}$ boats. Initial assumptions that all glycosidases harness $^4H_3$ conformations are incorrect; indeed, the utilisation of different transition states for the hydrolysis of glycosides is an emerging theme in glycobiology (Scheme 1)[3] and one that suggests a route for specific enzyme inhibition. Isofagomine lactam (**1**) displays an "in-plane" carbonyl at C2 and is, not surprisingly, a reasonable β-glucosidase inhibitor, with family GH1 β-glucosidases from *Thermotoga maritima* (*Tm*GH1) and sweet almond inhibited with K_i values of 130 nM (this work, Figure 1) and 29 μM,[4] respectively. Compound **1** has previously been shown to be an equally potent β-mannosidase inhibitor,[4] with the snail β-mannosidase inhibited with a K_i of 9 μM; this is superficially extremely counter-intuitive. Here, such K_i values for mannosidases are rationalised through structural analysis of **1** in complex with both an *exo* β-mannanase/β-mannosidase and a β-glucosidase. This work strongly supports previous proposals that β-mannosidases utilise a novel conformational itinerary, featuring a $B_{2,5}$ transition state. The ground-state axial O2 of mannose is thus pseudo-equatorial at the transition state in a way that should be harnessed in future generations of mannosidase inhibitors.



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Recently we described the conformational agenda of a retaining GH26 β-mannanase.[6] Trapping of the $^1S_5$ conformation for the "Michaelis" complex of unhydrolysed substrate, together with the $^OS_2$ conformation for the covalent intermediate, suggested a novel conformational itinerary for these enzymes through a $B_{2,5}$ transition state consistent with earlier proposals, notably by Sinnott[11] and Horton.[12] Glycoside hydrolases thus appear to be harnessing the full conformational itinerary in a way that is both enzyme and substrate dependent (this was recently reviewed in the context of inhibition by Vasella and colleagues).[13] Conformational considerations suggest that retaining mannosidase transition-state mimics should thus feature the pseudo-equatorial O2 of the $B_{2,5}$ conformation of mannose.

Isofagomine lactam **1**, synthesised by both Stick[14] and Bols,[4] contains an in-plane carbonyl at C2. In elegant work, Bols reports a K_i of 9 μM for the snail β-mannosidase;[4] this inspired us to study the three-dimensional structures of **1** bound to both *Tm*GH1 and a *Cellvibrio mixtus exo* β-mannanase (*Cm*Man5,[15] which is totally specific for *manno*-configured substrates). "Dual" inhibition of β-glucosidases and β-mannosidases has also been reported for the hydropyridazone compounds, which show similarity to isofagomine lactam, as discussed below.[16] The three-dimensional structures of *Tm*GH1 and *Cm*Man5 in complex with **1** were determined by X-ray

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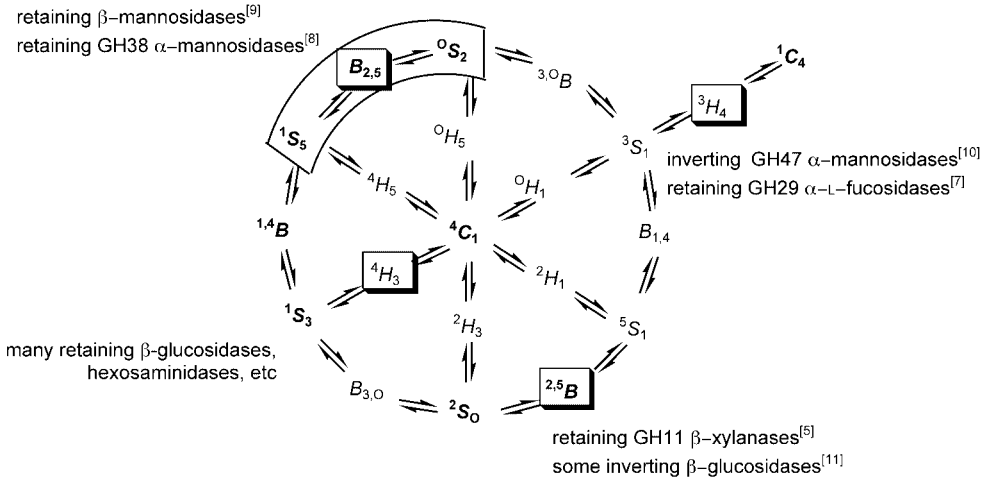


Scheme 1. *Pyranoside ring interconversion pathways. Potential transition states are shown in shadowed boxes, whilst relevant conformations observed "on-enzyme" in X-ray crystal structures are indicated in bold.[5,7–10] The proposed pathway for the hydrolysis of β-mannosides is shown boxed.*

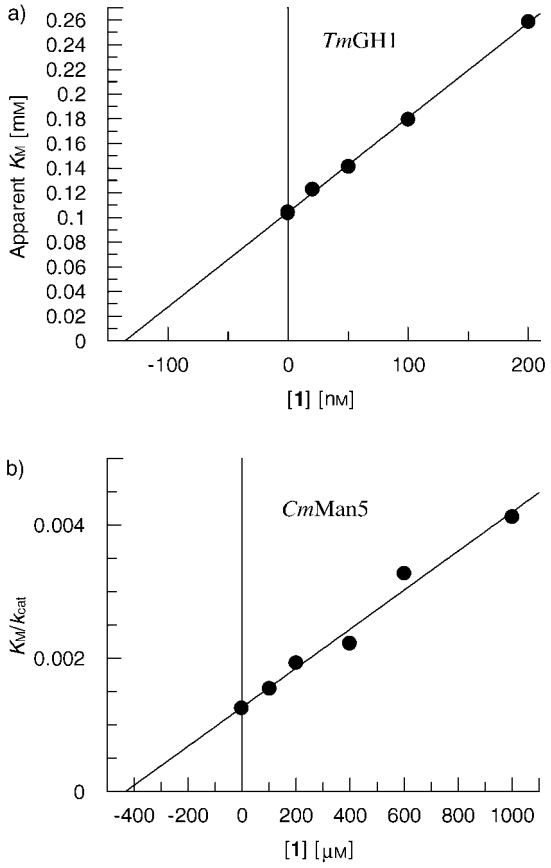


Figure 1. *K_i determinations for **1** bound to a)* Tm*GH1 and b)* Cm*Man5.*

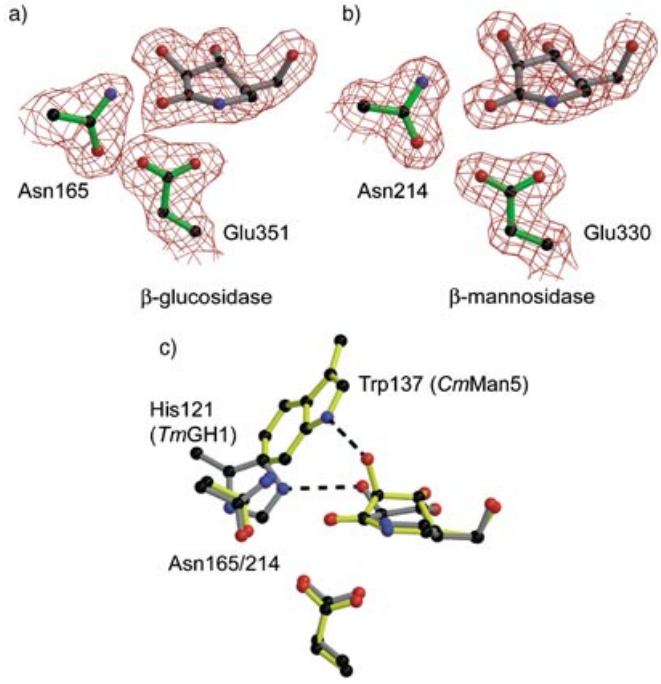
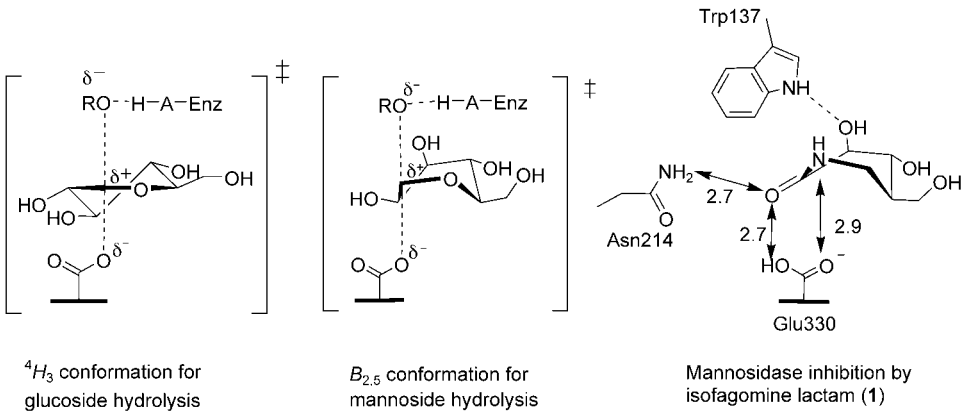


Figure 2. *Observed electron density (maximum likelihood/σ_A weighted $2F_{obs}-F_{calc}$ synthesis contoured at 1σ) for the binding of isofagomine lactam (**1**) to family 1 and 5 glycoside hydrolases. a) Binding of **1** to the* Thermotoga maritima *β-glucosidase* Tm*GH. b) Binding of **1** to the* Cellvibrio mixtus *β-mannosidase* Cm*Man5. The ligand is shown along with the catalytic nucleophile and an asparagine conserved in this "clan" of enzymes. c) Overlap of* Tm*GH1 (grey) and* Cm*Man5 (yellow) in complex with **1**; the primary residue interacting with O3 is shown.*

crystallography to resolutions of 2.0 and 1.7 Å, respectively, Figure 2a, b.

Both *Tm*GH1 and *Cm*Man5 are members of glycoside hydrolase "clan GH-A" and possess similar glycone (−1) subsites. In *Tm*GH1, **1** adopts its energetically favoured $^4H_5$ conformation as independently observed for the xylobiose-derived isofagomine lactam bound to two endoxylanases.[17,18] Such a conformation allows close interactions of O2 with Asn165 (2.9 Å to Nδ2) and the nucleophile (2.6 Å to Oε1). In *Cm*Man5, **1** is distorted away from its favoured $^4H_5$ conformation towards the $B_{2,5}$ conformation; C3, C4, N1 and C7 (equivalent to the O5 of mannose) form a reference plane with C5 and C2 lying 0.72 and 0.18 Å below this plane, respectively. Whilst interpretation of the conformation of **1** should clearly be considered with

caution (the glycone subsites of *Cm*Man5 cannot be harnessed by **1**, hence it displays a K_i value of ~400 μM[19] (Figure 1b), binding that is some 50 times weaker than that observed on a classical β-mannosidase[4]), it is extremely significant that a "glucose-like" piperidin-2-one, such as **1**, is able to inhibit enzymes that are totally specific for *manno*-configured substrates. The "in-plane" carbonyl of **1** permits the same close interaction of the nucleophile with O2 for β-mannosides (Scheme 2 and Figure 2b) as is well described for β-glucosidases and which, in the latter case, is believed to contribute significantly to catalysis.[20] Vasella and colleagues had previously postulated that the dual inhibition of β-mannosidases and β-glucosidases by the related di- and tetrahydropyridazinones might reflect an interaction between the carbonyl of the lactam moiety and the catalytic acid.[16] Such an interaction has not, so far, been observed in any of the relevant structures determined to date, and we would propose that the dual inhibition is related to the structure of the transition-state (either $B_{2,5}$ and $^4H_3$; Scheme 2) and not an adventitious interaction with the catalytic acid.



Scheme 2. *Different transition states for the enzymatic glucoside and mannoside hydrolyses allow inhibition by glucose-like lactams with "in-plane" carbonyl substituents at C2. The protonation state of the nucleophile shown for the complex with* ***1*** *is that determined experimentally from the 1.1 Å structure of the xylobio derivative of* ***1****.*[18]

Intriguingly, the overlap of the two structures shows that the most significant difference between the two potential conformational pathways is not at the 2-position, where O2 is pseudo-equatorial, but at the O3 position, which is pseudo-axial in the $B_{2,5}$ conformation (Figure 2c). In this context, the observations by Davis and colleagues that disruption of the 3-position interactions of a *Sulfolobus* family GH1 β-glucosidase changed its preference at the 2-position for *manno* versus *gluco*-configured substrates by a factor of 15 is extremely important.[21] Indeed, one may tentatively propose that the position of the residue interacting at the 3-position, His121 in *Tm*GH1 and Trp137 in *Cm*Man5 (Figure 2c), is a good predictor of transition-state conformation.

The $B_{2,5}$ and $^3H_4$ conformations now predicted for enzymatic β-mannoside hydrolysis alleviate the *cis* 1,2 interaction of mannose that bedevils synthetic chemistry, by placing O2 pseudo-equatorially at the transition state.[22] Intriguingly, the $B_{2,5}$ conformation is also that favoured by many *manno*-configured monosaccharide derivatives bearing an sp^2 anomeric centre.[12] Given the therapeutic potential for specific inhibition of various glycosidases, the harnessing of the diverse transition states by different enzymes is evolving into an area of huge potential. It is clear that future generations of inhibitor will either feature restraint into the appropriate conformation or tolerate conformational flexibility at low energetic cost. For the medically important endoplasmic reticulum/Golgi enzymes, the conformationally locked compound kifunensine ($^1C_4$ conformation and specific for $^3H_4$ transition state inverting α-mannosidases) is a flagship example of what may be achieved if conformational insight is harnessed for enzyme inhibition.[9] Recent observations of mannosidase inhibition by cyclic amidines revealed significantly better inhibition for compounds that likely favour the $B_{2,5}$ conformation;[23] this further demonstrates the practical exploitation of the structural principles described here. In this context, it will be important to observe the on-enzyme conformations and the role of O2 in the chemically diverse tight-binding mannosidase inhibitors synthesised by the Vasella group, with recent potent examples including isoquinuclidines[24,25] and tetrahydroimidazopyridines.[26]

Experimental Section

Enzyme kinetics and inhibition: Kinetic studies on *Tm*GH1 were conducted by monitoring the change in UV/visible absorbance with a Cintra 10 spectrophotometer, equipped with a Thermocell Peltier power supply. All experiments were performed at 37 °C, with a 5 min equilibration period for each sample prior to addition of *Tm*GH1. *Tm*GH1 activity was measured by using between 0.03 and 2 mM of 2,4-dinitrophenyl β-D-glucopyranoside as substrate and between 0 and 200 nM of **1**. All reactions were carried out in sodium citrate buffer (50 mM, pH 5.8) that contained BSA (1 mg mL^{-1}), in a total volume of 1 mL. The reaction was initiated by addition of *Tm*GH1 (10 μL) at a final concentration of 7 nM. 2,4-Dinitrophenolate release was monitored continuously at 400 nm for 300 s. Data for each inhibitor concentration were fitted to the Michaelis–Menten equation by using GraFit 5.0 (Erithacus Software Ltd, Horley UK) to obtain K_M and V_{max} values. The apparent K_M values at different inhibitor concentrations were plotted against the inhibitor concentration; the $-K_i$ was taken as the value at which the best-fit line crossed the *x* axis.

*Cm*Man5 was assayed in a 100 μL reaction volume consisting of sodium phosphate buffer (50 mM, pH 7.0) containing BSA (1 mg mL^{-1}) and the substrate 4-methylumbelliferyl β-D-mannopyranoside (5 mM). The reactions were initiated by the addition of enzyme to a final concentration of 200 nM and incubated for 4 h at 37 °C. At regular time intervals, 20 μL aliquots were withdrawn, and the pH was adjusted to 10.4 by the addition of glycine/NaOH

buffer (2 mL, 50 mM). The fluorescent product, 4-methylumbelliferone, was measured in a Shimadzu RF-150 L spectrofluorophotometer at 365 nm (excitation) and 460 nm (emission). As the concentration of substrate is approximately tenfold lower than the K_M, the reaction rate/substrate concentration gives the value of k_{cat}/K_M. Compound **1** was added to the enzyme reactions at concentrations between 100 μM and 1 mM; the $-K_i$ was taken as the value where the best-fit line of the plot of K_M/k_{cat} against inhibitor concentration crossed the *x* axis.

X-ray structure determination: Crystals of *Cm*Man5, prepared as in ref. [15], were incubated for 30 min with powdered **1**. Data, to 1.7 Å, were collected on beamline ID29 at the European Synchrotron Radiation Facility, and the structure was refined as described for the native enzyme.[15] Crystals of *Tm*GH1 were likewise soaked, and data were collected on beamline ID14-EH4 at the ESRF and refined as for the native enzyme.[27] All data were processed with the HKL suite.[28] Structures were refined with REFMAC,[29] and all other crystallographic computing used the CCP4 suite,[30] unless otherwise stated. Figures were prepared by using BOBSCRIPT[31] and MOLSCRIPT.[32]

Data files have been deposited at the Protein Data Base (www.pdb.org) with accession codes 1uz1 (*Tm*GH1) and 1uz4 (*Cm*Man5).

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