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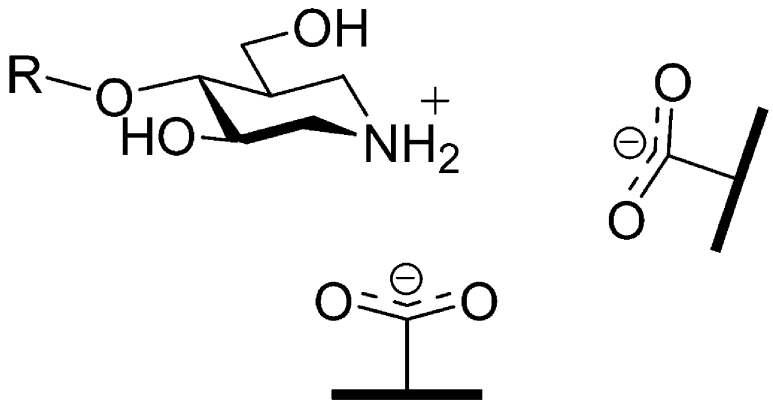

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Direct Observation of the Protonation State of an Imino Sugar Glycosidase Inhibitor upon Binding

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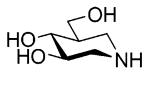
Glycoside hydrolases (hereafter glycosidases) are enormously efficient[1] enzymes that play a wide range of biological roles. Consequently, much interest has been expressed in the design, synthesis, and testing of potent inhibitors. Of particular importance are the tautomeric and protonation states of putative transition-state mimics and the surrounding enzyme, not only to inform strategies for therapeutic intervention but also to report on the nature of the transition state itself. Imino sugars are some of the most powerful inhibitors of glycosidase action, yet their degree, if any, of transition-state "mimicry" and their protonation state when bound to the target glycosidases remains unclear.[2] Furthermore, much of the published work assumes binding as the protonated form, but this has not been demonstrated directly.



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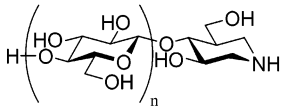

2, n=1; **3**, n=2; **4**, n=3

Here we present a detailed analysis of the inhibition of the endocellulase Cel5A from *Bacillus agaradhaerens* by a series of oligosaccharide glycosidase inhibitors (**2**−**4**),[3] derived from the parent isofagomine (**1**). The 1.0 Å resolution three-dimensional (3-D) structure of Cel5A in complex with **2** reveals that the imino sugar is protonated within the active site. Assignment of the protonation state of the catalytic apparatus in conjunction with the pH dependence of K_i and k_{cat}/K_M suggests that both the enzymatic nucleophile and the acid/base are unprotonated.

Cel5A is an endocellulase classified in "family GH5" of the CAZy classification.[4] Catalysis occurs with net retention of anomeric configuration,[5] via a covalent glycosyl enzyme intermediate, with Glu228 acting as the nucleophile and Glu139 as the acid/base.[6] The enzyme has a pH optimum of 6.0 (Figure 1) with acidic and basic limbs of pK_a 4.7 and 7.3, which most likely reflect titration of the catalytic nucleophile and acid/base, respectively. Crystals that diffract to 1.0 Å resolution are obtained at pH 5 where the enzyme retains approximately 75% activity, the loss reflecting partial protonation of the catalytic nucleophile. Indeed, previous atomic resolution analysis of snapshots along the reaction coordinate[7] confirms (partial) protonation of the nucleophile at low pH as reflected in the carboxylate CE-OE1 and CE-OE2 bond distances of 1.24 and 1.32 Å, respectively.

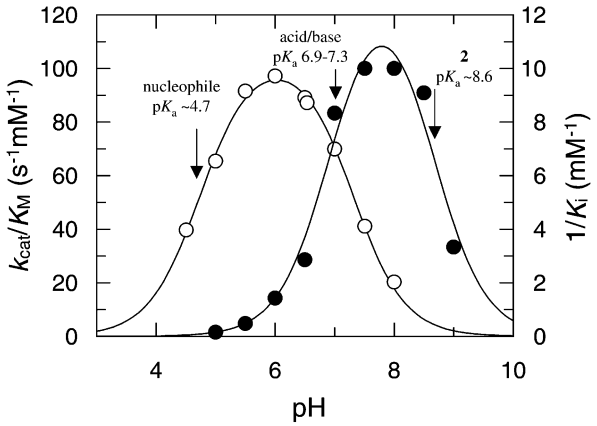

Figure 1. pH dependence of k_{cat}/K_M for Cel5A from *Bacillus agaradhaerens* (○) and $1/K_i$ for compound **2** (●). Calculated pK_a values and their putative origins are indicated.

Compounds **1**−**4** are competitive inhibitors of Cel5A with K_i values, determined at the pH optimum for catalysis, of 55 μM (**1**), 700 nM (**2**), 5 nM (**3**), and 400 nM (**4**).[8,9] The pH dependence of $1/K_i$ for **2** gives acidic and basic limbs with pK_a values of 6.9 and 8.6, respectively. The loss of binding at acidic pH most likely reflects titration of the acid/base, while the alkaline loss reflects titration of the isofagomine moiety (p$K_a \approx 8.4$[10]). On its own, the pH profile does not allow determination of the protonation state of the inhibitor since it is consistent both with binding of protonated inhibitor to an enzyme species in which both acid/base and nucleophile are deprotonated ($IH^+ + E$) and with the binding of unprotonated inhibitor to a protonated catalytic apparatus ($I + EH$).

The 3-D structure of Cel5A in complex with **2** was determined at 1.05 Å resolution (K_i **2** = 6.5 μM at pH 5).[11] Following maximum-likelihood refinement, $F_{obs} - F_{calc}$ "difference" electron density unambiguously reveals the position of all carbohydrate-associated hydrogen atoms. Compound **2** binds in the −2 and −1 subsites, with the isofagomine moiety in the catalytic −1 subsite[12] as expected (Figure 2). Both glucose and isofagomine moieties lie in undistorted $^4C_1$ (chair) conformations. Of most importance is the unambiguous observation that **2** binds to Cel5A as a protonated species (Figure 2), consistent with the pH dependence of $1/K_i$. The OE2 carboxylate oxygen of the nucleophile Glu228 lies 2.58 Å from the N1 of **2**, consistent with a close Coulombic interaction between the protonated isofagomine and negatively charged nucleophile (Figure 3). Electron density and geometrically unrestrained refinement reveal that the acid/base is likewise ionized, reflected in identical 1.26 Å C−O bond-lengths, and also forms a Coulombic

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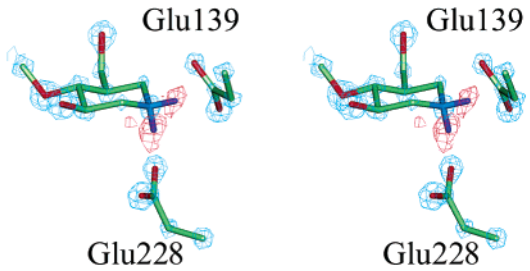

Figure 2. Observed electron density for inhibitor **2** in complex with Cel5A (only −1 site shown). $2F_{obs} - F_{calc}$, α_{calc} density at 2.2 eÅ^{-3} in blue and $F_{obs} - F_{calc}$ α_{calc} "difference" density at 0.1 eÅ^{-3} in red. The two hydrogen atoms visible at N1 are shown in purple.

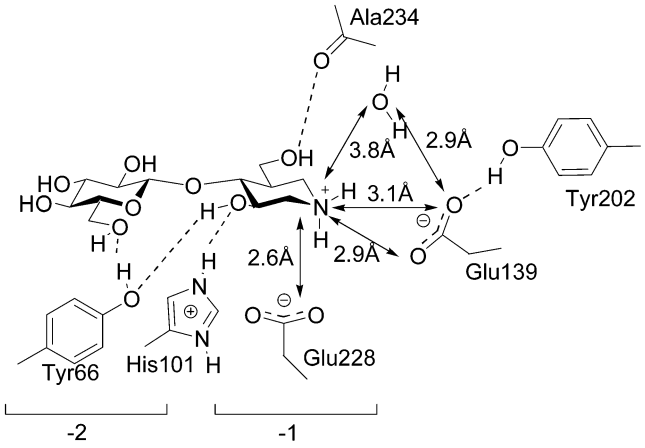

Figure 3. Schematic representation of the interactions observed between Cel5A and the cellobio-derived isofagomine (**2**). All hydrogen atoms shown have been observed experimentally. Only the −1 subsite interactions are shown in detail. Distances around N1 are indicated.

interaction with N1 of **2**. It is possible that at pH values below the pK_a of isofagomine the necessary deprotonation of the acid/base (and its potential structural consequences/solvent rearrangements) may contribute to the observed slow onset of inhibition. Also of note is a single water molecule 3.8 Å from N1 and hydrogen bonded to the acid/base (not shown in Figure 2), roughly approximating the position of the attacking water in the deglycosylation step of the reaction.[7] That the isofagomine moiety coordinates so few waters may contribute to the large favorable entropy reported for the binding of **1** on other systems.[13]

The implication of this atomic resolution structure, supported by the pH dependence of inhibition, is that **2** binds most tightly to a largely inactive enzyme species (Figure 1), with the two carboxylates clamping a protonated N1 (Figure 3). A similar "pincer-like" binding of a GalNAc isofagomine has been observed with the structure of the *Streptomyces plicatus β-N*-acetylhexosaminidase,[14] an enzyme utilizing acetamido-group participation, with a 2.7 Å interaction from the nucleophilic acetamido oxygen and a 2.8 Å interaction from the acid/base to the N1 position of the inhibitor. Likewise the 1.95 Å structure of the *Cellulomonas fimi* endoxylanase Cex, with a xylobio-derived isofagomine at pH 4.6, revealed a 2.6 Å association between the nucleophile and N1, although in this case the acid/base is much more distant.[15] While both structures were interpreted in terms of a protonated sugar, it is only with the benefit of atomic resolution data that the protonation of the isofagomine is confirmed.

The observed complex of protonated **2** could be interpreted as reflecting the charge distribution that occurs in the Cel5A transition state where the nucleophile and acid/base acquire partial negative charge and the substrate becomes oxocarbenium-like.[16] Weakly basic analogues of **1**, such as oxazine or 1-azafagomine, that would be expected to have more complementary pH profiles, are in fact weaker inhibitors of *β*-glucosidases than **1**.[17] Yet the observation that the tightest binding is to an inactive enzyme species, which cannot, by definition, bind the transition state, casts doubt on any interpretation based upon mimicry. Distortion of the isofagomine toward an oxocarbenium-like half chair is not observed. Indeed, both the position and $^4C_1$ conformation more closely resemble the covalent intermediate,[15] while the close association of the catalytic acid/base with N1 also hints that the potency of binding is partly fortuitous. The pK_a of **2** is not matched to those of the catalytic apparatus;[16] thus, the pH dependence of k_{cat}/K_M and $1/K_i$ do not correspond, placing **2** at odds with a criterion proposed for a transition-state analogues.[18] We believe that addressing such issues in future inhibitor design should not only produce more powerful inhibitors at catalytically relevant pH values but also allow targeting of specificity through pK_a matching.

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Supporting Information Available: X-ray methods with table of X-ray data and refined structure quality (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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