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Atomic resolution analyses of the binding of xylobiose-derived deoxynojirimycin and isofagomine to xylanase Xyn10A†

Tracey M. Gloster,[a] Spencer J. Williams,[b] Shirley Roberts,[a] Chris A. Tarling,[c] Jacqueline Wicki,[c] Stephen G. Withers[c] and Gideon J. Davies*[a]

[a] *Structural Biology Laboratory, Department of Chemistry, University of York, Heslington, UK YO10 5YW. E-mail: davies@ysbl.york.ac.uk*

[b] *School of Chemistry, University of Melbourne, Parkville, Australia 3010*

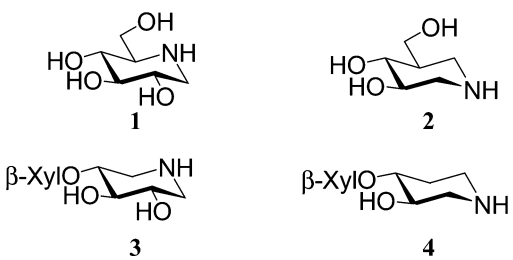
[c] *Department of Chemistry, 2036 Main Mall, University of British Columbia, Vancouver, Canada V6T 1Z1*

Received (in Cambridge, UK) 7th April 2004, Accepted 4th June 2004
First published as an Advance Article on the web 23rd July 2004

The atomic resolution structures of xylobiose-derived isofagomine and xylobiose-derived deoxynojirimycin in complex with the xylanase Xyn10A from *Streptomyces lividans* reveal undistorted $^4C_1$ chair conformed sugars and, in the case of the deoxynojirimycin analogue, suggest unusual pK_a changes of the enzyme's catalytic machinery upon binding.

The protonation state of imino-sugar glycosidase inhibitors and that of the catalytic centre to which they bind are central to their action. Imino-sugars such as 1-deoxynojrimycin (**1**) and isofagomine (**2**) are widely studied glycosidase inhibitors and it is commonly assumed that these inhibitors interact with the catalytic apparatus in a manner that has been interpreted either as mimicking the oxocarbenium ion-like transition state or simply being adventitious.[1] Whilst the former explanation seems intuitively reasonable, more quantitative analyses, such as the absence of a correlation between k_{cat}/K_M and $1/K_i$ for a series of modified substrates and inhibitors, favour the latter interpretation.[2] Regardless, most work assumes that these compounds bind in their protonated forms, but this has only recently been demonstrated through crystallography at "atomic resolution" with a cellobiose-derived form of **2**.[3] Here we present the "atomic resolution" structures of the *Streptomyces lividans* xylanase Xyn10A in complex with xylobiose-derived deoxynojirimycin (**3**) and isofagomine (**4**) at both pH 5.8 and 7.5. Electron density reveals the tight interaction of the NH group of **4** with the nucleophile Oε1 carboxylate oxygen, but the protonation states of both **3** and **4** remain unresolved despite atomic resolution data. pH profiles of inhibition are most consistent with the binding of protonated inhibitors to enzymes possessing both acid/base and nucleophile in a deprotonated form, but this interpretation is complicated by potential pK_a changes upon binding of the inhibitors, in particular **3**.

Xyn10A is a "classical" retaining glycosidase that performs catalysis *via* the formation and subsequent breakdown of a covalent glycosyl-enzyme intermediate, flanked by oxocarbenium ion-like transition states. Glu128 acts as the acid/base catalyst first protonating the leaving group to assist departure of the aglycon and then activating a solvent water molecule by deprotonation. Glu236 provides nucleophilic catalysis, which has been demonstrated through analysis of its long-lived 2-fluoroxylobiosyl and 2-fluorocellobiosyl enzyme intermediates.[4]



† Electronic supplementary information (ESI) available: kinetics and structural methods. See http://www.rsc.org/suppdata/cc/b4/b405152a/

The pH profile of catalysis for Xyn10A is bell-shaped with an optimum at 5.8 and acidic and basic limbs of pK_a 4.1 and 7.4, suggesting titration of the catalytic nucleophile and acid/base, respectively[5] (Fig. 1). Xyn10A is inhibited by **3** and **4** with K_i values (at pH 5.8) of 40 μM and 480 nM, respectively. The pH dependence of $1/K_i$ of **3** gives acidic and basic limbs of pK_a 6.6 and 7.8; a similar determination could not be made for **4** as the large alkaline shift in pH dependence, in agreement with other work,[3,6] did not allow fitting of the data and demonstrates maximal binding at high pH where the enzyme has lost all activity. Elsewhere this has been interpreted as protonated inhibitor optimally binding to an enzyme whose acid/base and nucleophile are both deprotonated.[3,6]

Crystals of Xyn10A for structural analysis were obtained both at pH 5.8 and 7.5 (the following descriptions refer to the pH optimum pH 5.8 data). Crystal structures with **3** and **4** were determined at around 1 Å resolution (supplementary information). Both **3** and **4** bind in the −2/−1 subsites as expected and the piperidine rings of the inhibitors are in $^4C_1$ (chair) conformations. At pH 5.8 **4** is well ordered in the crystal structure while **3** has an estimated occupancy of 0.75 (Fig. 2). Despite atomic resolution data, there is no observable difference density around N5 of **3** indicative of its protonation state, and the difference density for Xyn10A in complex with **4** reveals electron density only for the "α"-hydrogen that interacts with the nucleophile Oε1 atom. In contrast to previous work,[3] we cannot interpret the electron density in terms of protonation, or the lack thereof, because many ring and hydroxyl hydrogens on **3**, and to a lesser extent on **4**, are also absent in the difference density. The failure to observe hydrogen atoms with atomic resolution data is not uncommon and most likely reflects local mobility and disorder effects, discussed below.

Atomic resolution data do, however, allow very precise and unbiased positioning of the "heavy" atoms allowing bond lengths to be accurately determined and protonation states to be inferred. Glu236 (the nucleophile) in the pH 5.8 Xyn10A complex with **3** has considerable asymmetry with a bond distance of 1.30 Å from Cδ to Oε2 and 1.22 Å to Oε1 (Fig. 3) (the mean unrestrained carboxylate C–O distance is 1.26 Å, standard deviation 0.025 Å). In contrast,

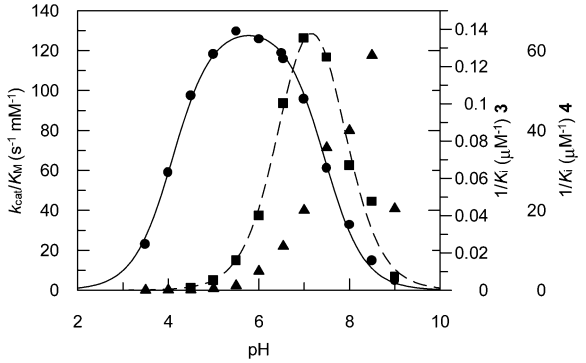


Fig. 1 pH dependence of k_{cat}/K_M for Xyn10A (●), $1/K_i$ for **3** (■) and $1/K_i$ for **4** (▲). Fits to "bell-shaped" profiles are shown for k_{cat}/K_M (solid line) and $1/K_i$ for **3** (dashed line).

DOI: 10.1039/b405152a

the data for the same complex at pH 7.5 yield similar bond lengths for Glu236 of 1.28 Å from Cδ to Oε2 and 1.24 Å to Oε1, indicating that the charge on the nucleophile is more delocalised and strongly suggesting that the nucleophile is protonated in the complex with **3** at pH 5.8, but deprotonated in the pH 7.5 complex. In the Xyn10A complex with **4** the Cδ–Oε bond lengths of the nucleophile are between 1.26 Å and 1.29 Å at pH 5.8 and 7.5 indicating the charge is largely delocalised in both cases. In the Xyn10A complexes with **3** or **4** at pH 5.8 or 7.5 the corresponding bond lengths for the acid/base residue (Glu128) are approximately equal suggesting that charge on this residue is delocalised, as has been observed previously.[8]

As many others have commented,[9] interpretation of pH profiles of inhibition is a risky pastime since they are composites of the pH-dependences of the free enzyme, the inhibitor and the EI complex. Compound **1** and its derivatives have proved especially troublesome with acid and basic limbs for $1/K_i$ difficult to interpret. The pH dependence of $1/K_i$ of **3** gives acidic and basic limbs of 6.6 and 7.8. Given the respective pK_a values (pK_a **3** 6.85; **4** 8.75; SGW and JW, unpublished) and the pH of crystallisation (5.8), it is expected that both **3** and **4** would be present in protonated forms. The data show that at pH 5.8, the nucleophile is protonated when **3** is bound

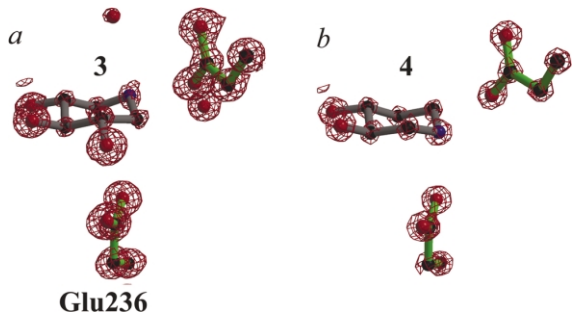


Fig. 2 Observed electron density for the binding of **3** (a) and **4** (b) to Xyn10A. 'Ball-and-stick' representation of the ligands with the nucleophile (Glu236) and acid/base (Glu128). Electron density for the maximum likelihood weighted $2F_{obs}–F_{calc}$ map is contoured at 1.5 e Å^{-3} ($\sim$2.5 σ) for **3** and at 2.5 e Å^{-3} ($\sim$3.75 σ) for **4**; figures were drawn using BOBSCRIPT[7].

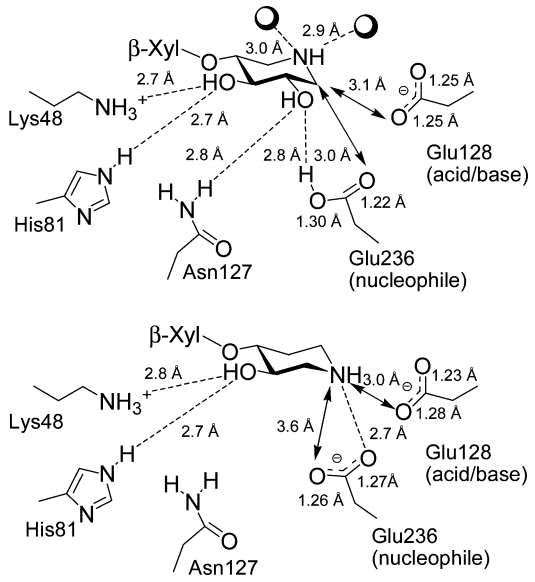


Fig. 3 Interactions of **3** and **4** with Xyn10A at pH 5.8. The protonation states of **3** and **4** could not be resolved by X-ray crystallography.

to Xyn10A. One interpretation of the pH profiles would be that the fall-off of $1/K_i$ with an acidic limb of 6.6 reflects titration of the nucleophile, whose pK_a has been raised approximately 2 units. Significant ligand-dependent shifts in the pK_a values for the carboxylates at the active centres of glycosidases are not unprecedented and have been directly measured on systems amenable to NMR.[10]

The marked difference in K_i for **3** and **4** may reflect not only the pK_a differences but also the thermodynamic contributions to binding reported on other systems.[6] These reports suggest that the tighter binding of **2**, relative to **1**, to β-glucosidases is derived solely from its more favourable entropy of binding. Zechel and colleagues have postulated that one contribution to this favorable entropy may be the difference in ordering of solvent water molecules upon complex formation[6] where the ordering of water molecules at molecular interfaces has been estimated to contribute between -0.5 and -3 kcal mol^{-1}. The 1 Å structures reported here are consistent with such effects for, whilst binding of **3** involves the coordination of two well-ordered water molecules to N5 (Figs. 2 and 3), the binding of the stronger inhibitor **4** involves no water-mediated contacts.

The work reported here emphasises both the advantages and shortcomings of atomic resolution crystallography. Whilst direct experimental observation of hydrogen scattering can be enlightening, failure to observe such density may reflect either the absence of hydrogen atoms or may instead result from other factors such as partial occupancy, mobility or local disorder.[11] The protonation states of both **3** and **4** when bound to enzyme thus remain ambiguous, although the protonation states of the enzymic nucleophile and acid/base are readily inferred. Both **3** and **4** bind in a "ground-state" $^4C_1$ conformation and, in the case of **4**, with an intimate hydrogen bond to Oε1 of Glu236. Again, here we observe that binding of the isofagomine derivative **4** is greatest at high pH where the enzyme has almost no catalytic activity, questioning its description as a "transition state mimic". It is clear that considerable work remains to be done on glycosidase transition state mimicry. Atomic resolution analyses, here and elsewhere[3,8] have shown that inhibitors may bind to active centers whose catalytic residues display different, often counter-intuitive, protonation states.

We thank the BBSRC, the EPSRC and PENCE for funding and Claude Dupont for provision of Xyn10A. G.J.D. is a Royal Society University Research Fellow.

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