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Transition-State Mimicry by Glycosidase Inhibitors: A Critical Kinetic Analysis

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Considerable interest has developed over the past decade in the design and synthesis of specific glycosidase inhibitors of high affinity.[1] A driving force has been the potential for such inhibitors as therapeutics, with successful examples being the neuraminidase inhibitors Tamiflu and Relenza (influenza)[2,3] and the α-glucosidase inhibitors acarbose and miglitol (diabetes).[4,5] Other inhibitors under development include those of mannosidases (cancer)[6] and of nucleoside hydrolases/phosphorylases.[7] In many cases, when high affinities are attained, the inhibitors are claimed to be transition-state analogues. However, high affinity by itself does not indicate transition-state mimicry, yet design of inhibitor modifications to improve affinity is frequently based upon such assumptions. In this paper, we describe an analysis of transition-state mimicry of two major classes of glycosidase inhibitors using a well-characterized enzyme system. We demonstrate that, at least with the enzyme chosen, aza-sugars bearing a formally sp^2-hybridized "anomeric center" are good transition-state mimics, whereas sp^3-hybridized aza-sugars of the deoxynojirimycin and isofagomine class, although equally potent, fit this description less well.

The xylanase Cex from *Cellulomonas fimi*, a "classical" retaining glycosidase that performs catalysis via oxocarbenium ion-like transition states, is an ideal test-bed for such an analysis since three-dimensional structures of the catalytic domain of this enzyme, of its covalent glycosyl−enzyme intermediate (Figure 1), and of complexes with a series of high affinity inhibitors have been described, along with numerous kinetic studies on wild-type and mutant forms.[8−14]

As described by Lienhard[15] and Wolfenden[16] and as reviewed by Mader and Bartlett,[17] a critical analysis of transition-state mimicry can be achieved by comparing the effects of equivalent structural perturbations on the affinity of the true transition state (via effects on substrate k_{cat}/K_m) and on the affinity of the transition state analogue (via K_i values). Two approaches may be used to introduce perturbations: (a) modifications to the inhibitor and the corresponding substrate and measurement of kinetic parameters with the wild-type enzyme, or (b) mutation of enzymic active site residues to afford mutant enzymes, which are studied with the same inhibitor and substrate. The second approach was used here. Ten different mutants representing modifications to eight active site residues (shown in red in Figure 1), including "first sphere" and "second sphere" side chains, were constructed. Kinetic parameters (k_{cat}, K_m, and k_{cat}/K_m) were determined for each of these mutants with the substrate *o*-nitrophenyl β-xylobioside at pH 7.0 (Table S1 of Supporting Information). Values of k_{cat}/K_m cover almost 3 orders of magnitude.

Five high affinity (K_i values between 0.13 and 5.8 μM) xylobiose-based inhibitors of Cex formed the basis for this study (Figure 2). Three of these (**1**, **2**, and **3**) possess sp^2-hybridized anomeric centers, thereby resembling the flattened anomeric stereochemistry at the transition state and, in the case of **1** and **2**, including an exocyclic heteroatom at C1 that mimics the protonated glycosidic oxygen. At the pH at which inhibition constants (K_i) were obtained (pH 7.0), **1** and **2** (conjugate acid pK_a values of 4.9 and 5.9) are neutral, but likely protonated by the enzyme. The other two aza-sugars, **4** and **5** (conjugate acid pK_a values of 6.9 and 8.8), are of the deoxynojirimycin and isofagomine types and are fully sp^3-hybridized with a $^4C_1$ conformation for both sugars. Three-dimensional structures of complexes of Cex with each inhibitor have been described.[12,13] K_i values for each of these inhibitors with each mutant are presented in Table S1 of Supporting Information.

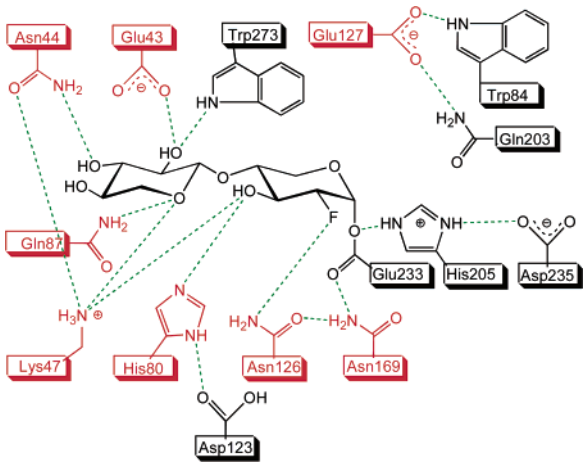

Figure 1. Schematic of the active site of Cex trapped as its 2-deoxy-2-fluoroxylobiosyl-enzyme intermediate.[18]

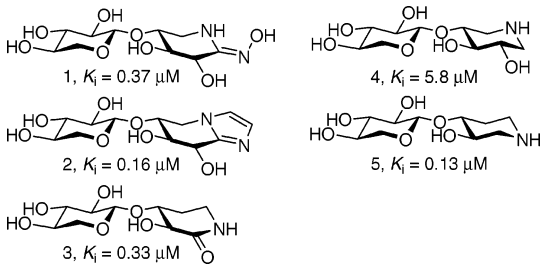

Figure 2. Structures of the five aza-sugar inhibitors studied, with K_i values against wild-type Cex xylanase shown.

To probe whether these inhibitors function as transition-state mimics, linear free energy relationships were plotted relating relative free energies of activation (from k_{cat}/K_m values for substrate hydrolysis) to free energies of inhibitor binding (from K_i values) as shown in Figure 3. Excellent correlations are observed for the three sp^2-hybridized inhibitors, with correlation coefficients from 0.97 to 0.99 and slopes of close to 1. Somewhat weaker correlations were seen for the isofagomine and deoxynojirimycin analogues, with correlation coefficients of 0.77 and 0.89 and slopes also close to 1. Importantly, no significant correlations were seen in log plots

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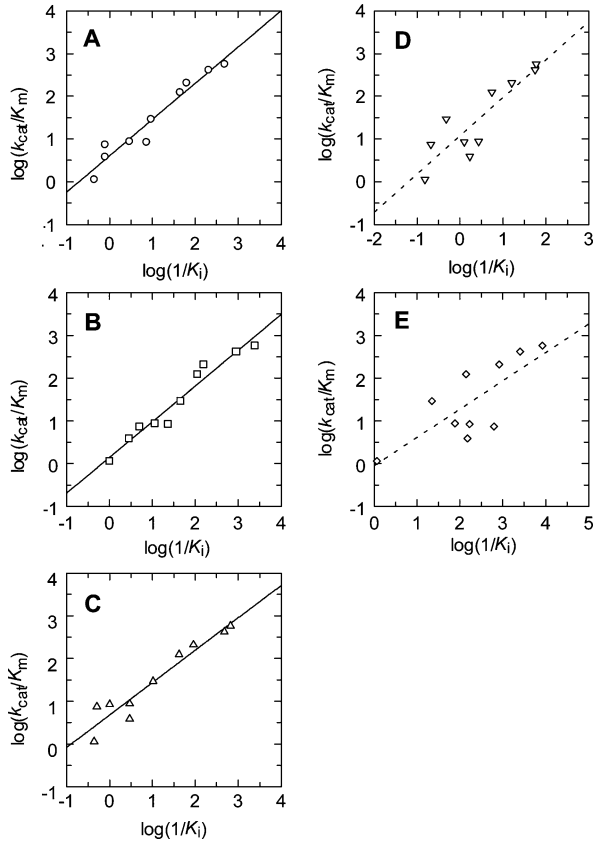

Figure 3. Transition-state analogy plots for *C. fimi* xylanase Cex. Comparison between k_{cat}/K_m values for hydrolysis of *o*-nitrophenyl β-xylobioside and inhibitor K_i values for xylobiose-based (A) lactam oxime **1**; (B) imidazole **2**; (C) isofagomine lactam **3**; (D) deoxynojirimycin **4**; and (E) isofagomine **5** with each of 10 mutants.

of K_i versus K_m (Supporting Figure 1), clearly showing that these are not simply ground-state effects.

These plots reveal differences in behavior of these two classes of inhibitors and the degree to which they reflect features of the transition state. An oxocarbenium ion-like transition state for this class of glycosidase, with the planar arrangement of C5, O5, C1, and C2 being accommodated within a half chair or envelope conformation, is strongly indicated by a number of studies, including heavy atom kinetic isotope effect analyses on a related clan member.[19] The excellent correlations for the sp^2-hybridized inhibitors (0.97−0.99) indicate that they quite accurately reflect the shape and charge of such a transition state. The poorer correlations observed for the sp^3-hybridized deoxynojirimycin (0.89) and isofagomine (0.77) type inhibitors indicate that while elements of their structure, presumably their charge, reflect the transition state other aspects, possibly their geometry, do not replicate the transition state as faithfully. The poorer correlation seen for the isofagomine **5** over the deoxynojirimycin **4** might reflect the absence of a 2-hydroxyl, which is known to contribute greatly to transition-state stabilization in β-glycosidases,[20] but could also reflect poorer mimicry of transition-state charge distribution. Indeed, this finding of poorer transition-state mimicry by **5** is reminiscent of conclusions of the calorimetric study on binding of isofagomine to the *Thermotoga maritima* β-glucosidase *Tm*GH1, where a large entropic contribution was detected, at odds with the normal requirement for enthalpic stabilization of the transition state.[21] The fact that the K_i value of the tightest binding inhibitor studied here, compound **2**, falls far short of the theoretical limit for a perfect transition-state analogue (estimated at 10^{-22} M)[22] reflects fundamental chemical limitations of transition-state mimicry: an inability to mimic the partial bonds to the nucleophile and nucleofuge and imperfection in van der Waals contacts. Strikingly, the poorest transition-state mimic studied here, the isofagomine **5**, binds to Cex as tightly as the best mimic, the imidazole **2**, demonstrating that powerful inhibition can be obtained even with limited resemblance of the transition state. While our results show that the sp^2-hybridized inhibitors appear to be the best transition state mimics for Cex, and presumably most other retaining glycosidases, recent studies have shown them to be poor mimics for retaining glycosidases that use substrate-assisted catalysis; NAG-thiazolines, which possess an sp^3-hybridized anomeric center, are better transition state mimics in that case.[23] Future design and elaboration of both sp^2- and sp^3-hybridized compounds as bespoke glycosidase inhibitors should be performed with these findings clearly in mind.

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Supporting Information Available: A table of the kinetic parameters measured, a log plot of substrate K_m values versus inhibitor K_i, complete ref 3, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Asano, N. *Glycobiology* **2003**, *13*, 93R−104R. (b) Bols, M. *Acc. Chem. Res.* **1998**, *31*, 1−8. (c) Heightman, T. D.; Vasella, A. T. *Angew. Chem., Int. Ed.* **1999**, *38*, 750−770. (d) Schramm, V. L. *Acc. Chem. Res.* **2003**, *36*, 588−596. (e) Ganem, B. *Acc. Chem. Res.* **1996**, *29*, 340−347.
(2) Moscona, A. *New Engl. J. Med.* **2005**, *353*, 1363−1373.
(3) von Itzstein, M.; et al. *Nature* **1993**, *363*, 418−423.
(4) Breuer, H. W. M. *Int. J. Clin. Pharmacol. Ther.* **2003**, *41*, 421−440.
(5) Scott, L. J.; Spencer, C. M. *Drugs* **2000**, *59*, 521−549.
(6) Goss, P. E.; Baker, M. A.; Carver, J. P.; Dennis, J. W. *Clin. Cancer Res.* **1995**, *1*, 935−944.
(7) Evans, G. B.; Furneaux, R. H.; Lenz, D. H.; Painter, G. F.; Schramm, V. L.; Singh, V.; Tyler, P. C. *J. Med. Chem.* **2005**, *48*, 4679−4689.
(8) MacLeod, A. M.; Tull, D.; Rupitz, K.; Warren, R. A. J.; Withers, S. G. *Biochemistry* **1996**, *35*, 13165−13172.
(9) Tull, D.; Withers, S. G. *Biochemistry* **1994**, *33*, 6363−6370.
(10) White, A.; Tull, D.; Johns, K.; Withers, S. G.; Rose, D. R. *Nat. Struct. Biol.* **1996**, *3*, 149−154.
(11) White, A.; Withers, S. G.; Gilkes, N. R.; Rose, D. R. *Biochemistry* **1994**, *33*, 12546−12552.
(12) Notenboom, V.; Williams, S. J.; Hoos, R.; Withers, S. G.; Rose, D. R. *Biochemistry* **2000**, *39*, 11553−11563.
(13) Williams, S. J.; Notenboom, V.; Wicki, J.; Rose, D. R.; Withers, S. G. *J. Am. Chem. Soc.* **2000**, *122*, 4229−4230.
(14) Williams, S. J.; Hoos, R.; Withers, S. G. *J. Am. Chem. Soc.* **2000**, *122*, 2223−2235.
(15) Lienhard, G. E. *Science* **1973**, *180*, 149−154.
(16) Wolfenden, R. *Nature* **1969**, *223*, 704−705.
(17) Mader, M. M.; Bartlett, P. A. *Chem. Rev.* **1997**, *97*, 1281−1302.
(18) Notenboom, V.; Birsan, C.; Warren, R. A. J.; Withers, S. G.; Rose, D. R. *Biochemistry* **1998**, *37*, 4751−4758.
(19) Lee, J. K.; Bain, A. D.; Berti, P. J. *J. Am. Chem. Soc.* **2004**, *126*, 3769−3776.
(20) Zechel, D. L.; Withers, S. G. *Acc. Chem. Res.* **2000**, *33*, 11−18.
(21) Zechel, D. L.; Boraston, A. B.; Gloster, T.; Boraston, C. M.; Macdonald, J. M.; Tilbrook, D. M. G.; Stick, R. V.; Davies, G. J. *J. Am. Chem. Soc.* **2003**, *125*, 14313−14323.
(22) Wolfenden, R.; Lu, X.; Young, G. *J. Am. Chem. Soc.* **1998**, *120*, 6814−6815.
(23) Whitworth, G.; Macauley, M.; Stubbs, K.; Dennis, R.; Taylor, E.; Davies, G. J.; Greig, I. R.; Vocadlo, D. J. *J. Am. Chem. Soc.* **2007**, *129*, 635−644.

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