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Analysis of the Reaction Coordinate of α-L-Fucosidases: A Combined Structural and Quantum Mechanical Approach

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Human glycans including N-linked glycans, ABO blood group and Lewis antigens, are decorated with terminal α-L-fucose residues. In man, the dynamic process of α-L-fucoside (**1**) removal is catalyzed by two α-L-fucosidases (FucA1 and FucA2) which liberate α-L-fucose (**2**) as a product (Figure 1).[1,2] These enzymes are important in regulating bacterial infections,[1] and a deficiency in these enzymes leads to fucosidosis, a debilitating neurodegenerative lysosomal storage disorder.[3,4] Some cancers are identified by increased fucosylation levels on their surface, which promotes growth and metastasis of cancerous cells.[3,4] Increased levels of α-L-fucosidase expression has been important in cancer prognosis[5,6] and is a potential target for cancer therapy.[7] Understanding the catalytic mechanism of α-L-fucosidases is central to an understanding of their function.

Previous work on these enzymes, which act with net retention of the anomeric configuration, postulated a $^3S_1$ covalent intermediate providing the first insight into their conformational itinerary.[8] Here, using a glycoside hydrolase (www.cazy.org)[9] family GH29 fucosidase, Bt2970 from *B. thetaiotaomicron* which shows high homology to human enzymes, we describe a series of snapshots of the reaction coordinate of these enzymes. The Michaelis complex is observed in a $^1C_4$ conformation, a trapped covalent intermediate in the $^3S_1$ skew boat which together provides structural evidence for a "latitudinal" Southern Hemisphere $^1C_4 \leftrightarrow {}^3H_4 \leftrightarrow {}^3S_1$ pathway for terminal α-L-fucoside hydrolysis by this family of enzymes (glycosidase mechanisms have recently been reviewed in ref 10). Evidence for this pathway is supported by quantum calculations, which reveal that the $^1C_4$ conformer is also the best activated for catalysis in terms of energy, anomeric charge, and bond length contraction/elongation and that $^1C_4 \leftrightarrow {}^3H_4 \leftrightarrow {}^3S_1$ is a favored itinerary. Results aid the interpretation of inhibitor binding, exemplified here by an α-L-fucose-like iminosugar that mimics the $^1C_4$ ES complex by mimicking the charge distribution across O5−C1. Collectively, the results of this study define the conformational itinerary of GH29 fucosidase catalysis and should aid the design of further generations of inhibitors as chemical probes and potentially as therapeutic agents.

The native structure of *Bt*Fuc2970 was initially solved at 1.7 Å resolution from a $P2_1$ crystal form (Supporting Information, SI). Subsequently this was used as the starting model for complex structure analysis. A "Michaelis" complex for *Bt*Fuc2970 was obtained using an enzyme variant in which the catalytic nucleophile had been crippled through construction of the aspartate to asparagine substitution (D229N), an approach that had worked on related systems (for example[11,12]). This inactive enzyme was used in conjunction with the aryl glycoside substrate 4-nitrophenyl α-L-fucoside **3** which was soaked into crystals of the D229N variant for ~5 min prior to freezing and data collection (SI). The observed X-ray data at 1.95 Å resolution revealed an unambiguous electron density for the unhydrolysed substrate **3**, which was observed in the $^1C_4$ conformation, Figure 2a,b. Features of the complex include (albeit with the caveat that this is an Asp to Asn variant) a nucleophile−C1 distance of 3.4 Å and an $O_{nuclophile}$−C1−$O_{leaving\ group}$ angle of ~160°, consistent with in-line nucleophilic attack (discussed below). Furthermore, the 3-D structure strongly supports the identification of the catalytic acid proposed by Sulzenbacher and colleagues[8] (equating here to Glu288); a structural feature conserved in all four known 3-D structures of GH29 fucosidases (this work and PDB codes 1hl9,[8] 3eyp and 3gza) but apparently absent in the human enzymes.[2] There remains a conundrum that, on the basis of sequence alone, neither the catalytic acid observed structurally nor the human residue proposed through kinetic analysis of variants is conserved within the family. Adding to the complexity, it is possible that the human Glu308 variant (that shows many of the phenotypes of a catalytic acid variant adding to confusion) effects its action through a series of interactions to the true acid, and one may speculate that human Glu308 variants thus perturb alternative acids, *en passant*.

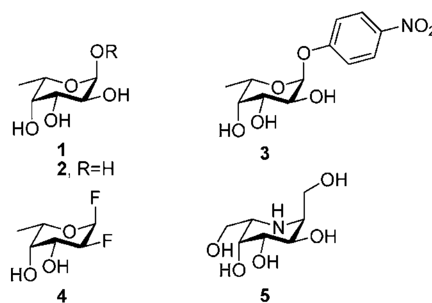

Figure 1. Structures of a generic α-L-fucoside (**1**), α-L-fucose (**2**), and compounds used to study fucoside hydrolysis described in this paper.

Access to the covalent intermediate was facilitated through use of the 2-fluoro fucosyl fluoride **4** (synthesis described in ref 13). On the wild-type enzyme, the expected 2-fluoro fucosyl-enzyme intermediate was too rapidly turned over to allow conventional trapping and so the acid/base variant E288Q was further used to reduce the deglycosylation rate (also providing support for the acid/

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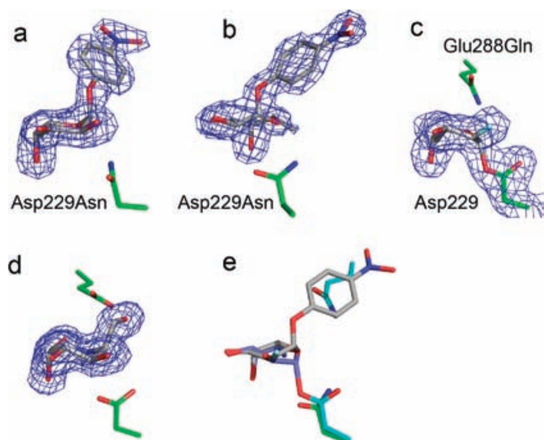

Figure 2. Observed electron density and 3-D structures for complexes of the family GH29 fucosidase, Bt2970. (a) Michaelis complex of the D229N variant with **3** "side-on and (b) "end-on". Panel (c) shows the trapped covalent 2F fucosyl-enzyme intermediate in $^3S_1$ conformation. Panel (d) shows the iminosugar inhibitor **5**. (e) The overlap of Michaelis and Intermediate complexes shows how catalysis can be envisaged of a small electrophilic migration of the anomeric carbon. Electron densities shown are $2F_{obs} - F_{calc}$ maps contoured at ~0.4 e$^-$/Å^3.

base assignment discussed previously) to permit accumulation of the covalent intermediate prior to cryo-trapping of the reacted crystal. In this manner, the fucosyl-enzyme intermediate was trapped on the E288Q variant and subsequently analyzed by X-ray crystallography, at 2.1 Å. Again, the observed electron density unambiguously reveals the trapped β-L (2F) fucosyl enzyme intermediate, Figure 2c, here in the $^3S_1$ (skew boat) conformation with the β-linkage to the nucleophile Asp229. GH29 enzymes are "*syn*" protonators,[14] and consistent with proposals by Nerinckx and others[15] no "third" carboxylate is seen in the active center interacting with the endocyclic oxygen.

The trapped Michaelis and glycosyl-enzyme intermediate complexes strongly support the "Southern Hemisphere" conformational itinerary for GH29 enzymes, as originally proposed by Sulzenbacher and colleagues on the basis of the trapped intermediate complex alone.[8] In this itinerary in-line nucleophilic attack on the α-L-fucoside substrate (in $^1C_4$ conformation), with its axial leaving group orientation, results in "electrophilic migration" of the anomeric carbon, Figure 2e, through the $^3H_4$ at (or close to) the likely transition state to the intermediate in the $^3S_1$ conformation, Figure 3. Hence one can consider a $^1C_4 \leftrightarrow {}^3H_4 \leftrightarrow {}^3S_1$ "latitudinal" pathway for the formation of the covalent glycosyl-enzyme intermediate.

To probe this conformational pathway further, first principles metadynamics simulations[16] on isolated α-L-fucose were performed (details in the SI). The conformational free energy landscape, Figure 4, shows that the $^1C_4$ conformation for α-L-fucose is indeed the lowest in free energy and that a second energy minimum also occurs in the $^3S_1$ region,[17] as occupied by the covalent intermediate structures observed here and previously. As with a previous study on β-D-glucose,[18] the conformational free energy landscape of the isolated sugar alone is entirely consistent with the conformations of enzyme–ligand complexes observed by X-ray crystallography.

Further analysis of structural and electronic properties of α-L-fucose conformations of (see SI) reveals that the $^1C_4$ conformer is the one better prepared for catalysis in terms of free energy, C1–O1/O5–C1 bond length elongation/reduction, C1–O1 bond orientation, and positive charge development around the anomeric carbon (Figures S2–S4 and Table S1).

The preactivation of the $^1C_4$ conformation, in particular the increased positive charge across O5–C1, provides additional impetus for the analysis of iminosugar inhibitors. We took advantage of one such compound, **5**, recently reported by several groups,[19] with the belief that the positive charge at the secondary nitrogen center would mimic the preactivated substrate complex and related flanking transition state. Isothermal titration calorimetry indeed shows that **5** is a reasonably tight binding *Bt*Fuc2970 inhibitor with a K_d of 755 (±55) nM at pH 6.0 (Figure S1), compared to a K_d for fucose of 230 (±7) μM obtained by ITC in the same buffer. The structure of **5** in complex with the enzyme was therefore subsequently solved at a resolution of 1.8 Å, Figure 2d. As expected, **5** binds in the $^1C_4$ conformation with the additional O6 hydroxyl moiety not making any direct contacts with the protein; instead it occupies a gap within the active site surrounded by residues F64, W227, C308, and W316. The hydroxyl methyl group at C1 forms hydrogen bonds with the catalytic acid E288 and an ordered water molecule which is positioned to serve as the nucleophile in the deglycosylation step of hydrolysis. The endocyclic amine hydrogen bonds with Oε2 of nucleophile D229 while C2, C3, and C4 OH groups hydrogen bond with H136, H135, and H66 respectively, similar to the interaction observed with the fucosyl moiety in **3**. Thermodynamically, **5** is enthalpically similar to fucose but makes a more favorable entropic contribution (see SI).

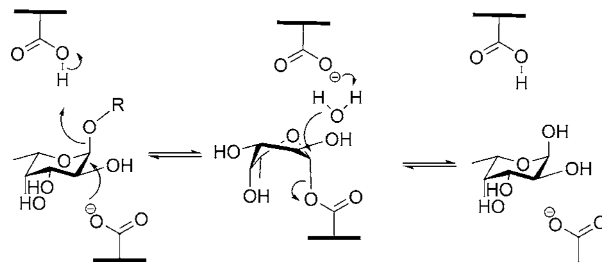

Figure 3. Likely reaction coordinate for GH29 α-L-fucosidase catalyzed hydrolysis of fucosides. The Michaelis complex (Figure 2a) binds in the $^1C_4$ conformation affording unhindered in-line nucleophilic attack (electrophilic migration) of the anomeric carbon to the intermediate in the $^3S_1$ conformation which is subsequently deglycosylated by water.

A $^1C_4 \leftrightarrow {}^3H_4 \leftrightarrow {}^3S_1$ pathway for the glycosylation step of the α-L-fucosidases of family GH29 adds to an emerging picture for glycosidase catalysis in which diverse enzymes harness different conformational pathways (reviewed in ref 10) building on concepts first expanded upon by Sinnott.[20] So far, "Southern Hemisphere" pathways (considering Pople and Cremer[21a] and Stoddart[21b] definitions) are comparatively rare (likely reflecting, in part, the paucity of L-glycosides, and thus L-glycosidases, in nature). A $^3S_1 \leftrightarrow {}^3H_4 \leftrightarrow {}^1C_4$ for GH47 inverting α-D-mannosidases has considerable experimental support[22,23] as does an analogous pathway for the glycosylation step of neuraminidase-catalyzed hydrolysis of sialic acid containing ketals (exemplified by refs 24 and 25).

In the case of GH29 catalyzed hydrolysis, the $^1C_4 \leftrightarrow {}^3H_4 \leftrightarrow {}^3S_1$ is highly analogous to a $^4C_1 \leftrightarrow {}^4H_3 \leftrightarrow {}^1S_3$ glycosylation itinerary for a D-*gluco* configured substrate as proposed for some retaining α-D glycosidases, notably the GH31 α-xylosidases (covalent intermediate[26] in $^1S_3$ and Michaelis complex[27] in $^4C_1$), and for retaining GH27 α-galactosidases,[28] likewise. Thus GH27, GH29, and GH47 retaining enzymes all appear to harness pathways that are the "reverse" of related β-glycosidase pathways (thus displaying skew boat conformed intermediates) while GH13 cyclodextrin transglycosylases[29] and α-amylases[30] do not; their trapped intermediates instead adopt $^4C_1$ conformations.

Recent computational analyses (for example[31–33]) have disentangled the interplay of electronic/structural factors behind substrate preactivation in GHs. Excitingly, what is becoming apparent, initially, for β-D glucose[18] and now α-L-fucose is that the chemical

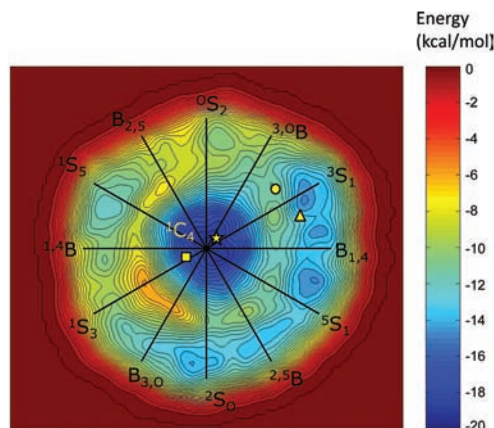

Figure 4. Computed free energy landscape of α-L-fucose with respect to ring distortion (southern hemisphere of the Cremer−Pople[21a] sphere). Each contour line of the diagram corresponds to 0.5 kcal mol^{-1}. Symbols plot the observed conformations: Fucosyl-enzyme intermediates on BtFuc2970 (filled triangle, this work) and on the *Thermatoga* GH29[8] (filled circle), the PNP α-L-fucoside Michaelis complex (this work, filled square), and the imino-sugar inhibitor **5** (this work, star).

aspects of the isolated sugar alone predict a small number of possible conformational pathways, reflected in lower free energy states and favorable "preactivation" for catalysis, but that individual enzyme families select only one of these theoretical possible routes. In that light, it will be interesting to see if other families of enzymes acting on fucosides harness the pathways around the $B_{1,4}$, $^{5}S_1$, $^{2}S_O$ area of the conformational hemisphere, Figure 4, which are also energy minima. This enzymatic selection of a single pathway presumably reflects the evolutionary origin of the given enzyme and thus its disposition of active center groups. The combination of first principles molecular dynamics and structural approaches[34] offers increased potential for exploitation to design and harness small molecule chemical probes as has so elegantly been demonstrated by Schramm in the case of purine nucleotide phosphorylases, for example.[35−37]

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Note Added in Proof. Readers of this paper will be interested in a related paper on the inhibition of GH29 fucosidases which has appeared during the preparation of this article. Wu and colleagues (Wu, H.-J.; Ho, C.-W.; Ko, T.-P.; Popat, S. D.; Lin, C.-H.; Wang, A. H.-J. *Angew. Chem. Int. Ed.* **2010**, *49*, 337−340) describe the 3-D structures of the *Thermotoga maritima* α−L fucosidase with micro-picomolar enzyme inhibitors.

Supporting Information Available: Table of X-ray data and structure quality; experimental methods including X-ray analyses, and details of the metadynamics simulations; ITC data). This material is available free of charge via the Internet at http://pubs.acs.org.

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