Enzyme Models

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Substrate Distortion by a Lichenase Highlights the Different Conformational Itineraries Harnessed by Related Glycoside Hydrolases**

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The conformational itineraries, adopted by the more than twenty thousand glycosidases, present an emerging picture that these enzymes adopt a variety of specific reaction pathways.[1] Catalytic itineraries for the enzymatic hydrolysis of glycosides likely feature one of four transition-state pyranoside-ring conformations: one of two classical boats ($B_{2,5}$ and $^{2,5}B$) or one of two half chairs ($^{4}H_{3}$, $^{3}H_{4}$, and/or their closely related envelope forms ^{4}E and ^{3}E).[2] Considerations of the conformational aspects of the transition state are becoming central to the development of specific transition-state mimics both as mechanistic and cellular probes and as therapeutic agents.[3] A central question is raised by these conformational pathways: is the (on-enzyme) transition-state structure dictated solely by the chemistry of the parent glycoside, the topography of the enzyme active centre, or a subtle interplay of both? The glycoside hydrolase family GH26 provides a powerful system to investigate these issues as, although most of its members are β-mannanases,[4] it has recently been shown that select GH26 enzymes are specific for "gluco-configured" substrates, notably β-1,3:β-1,4 mixed-linkage β-glucan lichenan[5] and β-1,3 linked xylan.[6] Herein we present a series of enzymatic snapshots along the reaction coordinate of a family GH26 lichenase from *Clostridium thermocellum*, hereafter named *Ct*Lic26A. The Michaelis complex of this enzyme with unhydrolyzed methylumbelliferyl β-laminaribioside **1** reveals a conformation between $^{1}S_{3}$ (skew) and $^{1,4}B$, whereas the covalent reaction intermediate, trapped through the use of the 1,2-difluoro trisaccharide **2**, is

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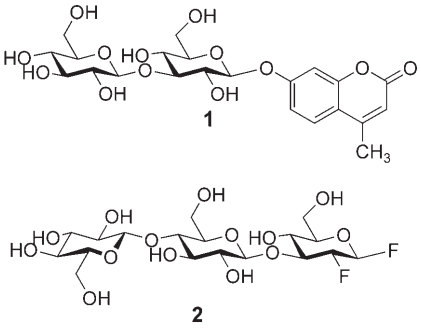
found in the $^4C_1$ (chair) conformation. These conformations are in stark contrast to the $^1S_5$ and $^{O}S_2$ conformations observed for the equivalent complexes of the closely related β-mannanases from this family.[4] These observations strongly suggest that individual, even closely related, enzymes harness different conformational itineraries that are necessarily a composite both of enzyme structure and glycoside chemistry.



*Ct*Lic26A is a lichenase whose favored substrates are mixed linkage β-1,3:β-1,4 glucans.[5] Catalysis occurs with net retention of the anomeric configuration through the formation (glycosylation) and subsequent breakdown (deglycosylation) of a covalent glycosyl-enzyme intermediate that is flanked on either side by oxocarbenium-ion-like transition states. Such a reaction demands two essential catalytic groups: a nucleophile and an acid/base that, in this family, are both enzyme-derived carboxylates. The sequence of the enzyme is classified into the glycoside hydrolase (GH) family 26, a family more normally associated with β-mannanases. This classification remained even though the 3D structure[5] has shown it to be a "clan GH-A" enzyme[7] with a characteristic $(\beta/\alpha)_8$ barrel and the signature catalytic constellation of catalytic acid/base and nucleophile on strands β-4 and β-7, respectively. The Michaelis complex of *Ct*Lic26A was trapped by using methylumbelliferyl-β-laminaribioside **1** in conjunction with an inactive enzymatic variant in which the catalytic nucleophile Glu 222 was replaced by glutamine: cocrystallization of Glu 222 Gln *Ct*Lic26A with **1** afforded the Michaelis complex with the X-ray structure solved to 1.4 Å resolution. Similarly, preincubation of the acid/base Glu 109 Ala mutant of *Ct*Lic26A with the 1,2-difluoro trisaccharide **2**, followed by crystallization, allowed crystallographic observation of the trapped 2-fluoroglycosyl enzyme intermediate, which was also observed at a resolution of 1.4 Å.

The structure of the Michaelis complex clearly reveals the unhydrolyzed substrate, at full occupancy, distorted into an approximate $^1S_3$ conformation with an axial leaving group, fulfilling the stereoelectronic requirements of an incipient oxocarbenium-ion-like species and with the "nucleophile" poised, in what might be termed a "near-attack conformation",[8] for in-line attack at the anomeric centre, Figure 1. Such a structure is also consistent with the dictates of the antiperiplanar lone-pair hypothesis.[9] Similarly, the covalent glycosyl-enzyme intermediate, stabilized both through the 2-fluoro substituent and by the lack of an appropriate catalytic base for the deglycosylation step, lies in a relaxed $^4C_1$ conformation, Figure 1.

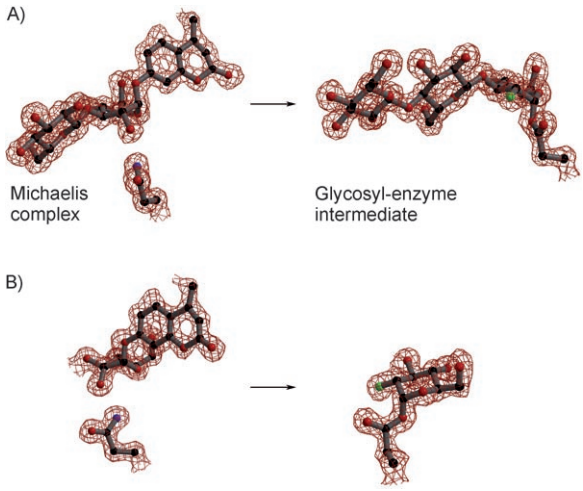
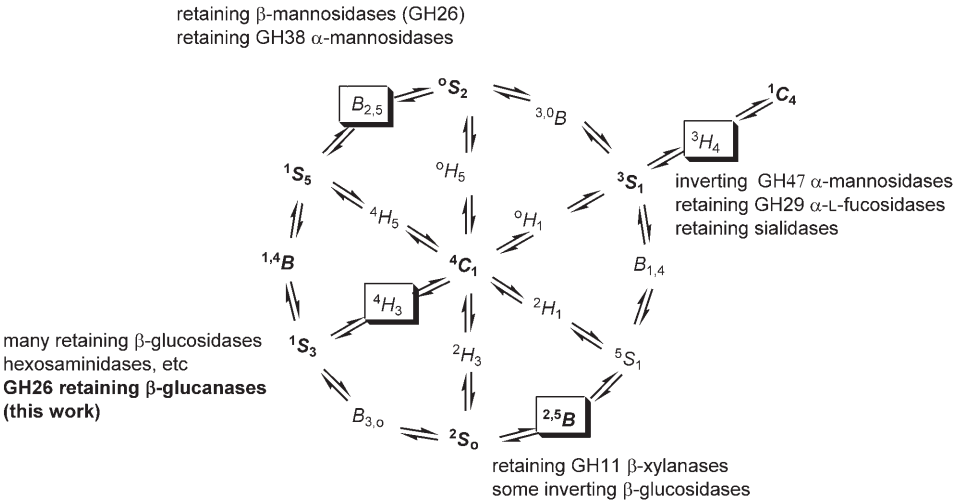


Figure 1. Side-on (A) and end-on (B) views of the trapped Michaelis and glycosyl-enzyme intermediate complexes for *Ct*Lic26A. For the end-on views, only the −1 subsite pyranoside is shown for clarity, together with the methylumbelliferyl group for the Michaelis complex. Electron density shown is a $2F_{obs}-F_{calcd}$ synthesis contoured at approximately 1σ (0.55 electrons/Å^2).

These two structures, representing "snapshots" along the glycosylation reaction coordinate of *Ct*Lic26A, are similar to those observed previously for some GH5 (also Clan GH-A) β-glycosidases active on gluco-configured substrates.[10] They are thus consistent with a $^4H_3$ (or the closely related ^4E) conformation for the transition state, which is flanked by the $^1S_3$ and $^4C_1$ conformations in the classical pyranoside interconversion agenda, Scheme 1. Catalysis is thus likely to occur through a simple "electrophilic migration"[11] of the anomeric carbon along the reaction coordinate. Notably, however, the $^1S_3 \rightarrow {}^4H_3 \rightarrow {}^4C_1$ migration for the family GH26 lichenase is not observed in β-mannoside hydrolysis by β-mannanase *Cj*Man26A (β-mannanase is the predominant activity displayed by GH26 enzymes) from this family.[4]

Previously, X-ray crystallography had been used to take a series of enzymatic "snapshots" along the reaction coordinate of *Cj*Man26A. Observation of an unusual $^1S_5$ conformation for the Michaelis complex of unhydrolyzed substrate suggested that the conformationally adjacent $B_{2,5}$ conformation might be that adopted by the reaction transition state. Subsequent trapping of the covalent reaction intermediate in an $^{O}S_2$ conformation lent strong credence to this proposal as $^1S_5$ and $^{O}S_2$ conformations flank $B_{2,5}$ in the pyranoside interconversion agenda, see Scheme 1. Catalysis is again considered as an "electrophilic migration" of C1, through a $B_{2,5}$ transition state, consistent with the principle of least molecular motion. Furthermore, it was evident that such a transition state for enzymatic mannoside hydrolysis has an additional advantage in that it places O2 pseudoequatorial, therefore relieving unfavorable 1,2 diaxial effects for nucleophilic substitution at the anomeric carbon. Additional evidence for the relevance of the $B_{2,5}$ conformation comes from the observations that D-mannono-1,5-lactone and 5-



Scheme 1. Pyranoside interconversion itinerary with likely reaction coordinates for the different glycosidases shown. Potential transition states are shown boxed and conformations observed "on-enzyme" in crystal structures are indicated in bold.[1,12] Retaining sialidases are believed to use what equates to the $^{3}S_{1}\rightarrow{}^{3}H_{4}\rightarrow{}^{1}C_{4}$ pathway ($^{5}S_{2}\rightarrow{}^{4}H_{5}\rightarrow{}^{2}C_{5}$).[13]

amino-5-deoxy-D-mannono-1,5-lactam, both sp^2 hybridized at C1, also favor this unusual boat conformation.[14] The proposal of a $^{1}S_{5}\rightarrow B_{2,5}\rightarrow{}^{O}S_{2}$ conformational change for a retaining β-mannanase predicted both that retaining α-mannosidases could utilize a "reverse" conformational agenda ($^{O}S_{2}\rightarrow B_{2,5}\rightarrow{}^{1}S_{5}$) and that these enzyme classes should be inhibited by compounds featuring "pseudoequatorial" groups at C2. Subsequently, the β-linked covalent intermediate for the family of GH38 retaining α-mannosidases has indeed been observed in $^{1}S_{5}$ conformation[15] and likewise, a significant number of compounds with "pseudoequatorial" C2 substituents have been shown to be inhibitors of β-mannosidases.[12,14,16]

One of the important, indeed controversial, aspects of glycosidase catalysis is whether sequence and structurally related enzymes working on different glycoside substrates adopt similar, or markedly different, conformational approaches to catalysis; does the enzyme, the substrate, or a subtle interplay of both dictate the catalytic itinerary and transition-state conformation? Comparison of the family GH26 β-mannanases and lichenses that are specific for manno- and gluco-configured substrates, respectively, in the light of wider work on other glycosidase families, suggests that the conformational agenda adopted is necessarily a subtle interplay of enzyme-structure-derived geometric constraints and the stereoelectronic and steric dictates of the substrate. X-ray crystal structure observations have certainly revealed that structurally, and therefore evolutionarily, related enzymes do not necessarily adopt the same catalytic pathways. The geometry of the substrate alone, however, does not dictate its reaction pathway; there is strong evidence that inverting α-mannosidases (family GH47) instead harness a $^{3}H_{4}$ transition state,[17,18] whereas some inverting β-glucosidases are believed to harness a $^{O}S_{2}\rightarrow{}^{2,5}B$ pathway (for an example, see reference [19]). For β-xylanases, we again see employment of different conformational pathways: GH10 enzymes are believed to perform catalysis through a $^{4}H_{3}$ transition state, whereas structurally-unrelated GH11 enzymes may harness a $^{2,5}B$ conformation,[20] see Scheme 1. Given the increasing number of examples, we propose that structurally related enzymes working on similar substrates are likely to harness the same conformational pathways, but that related enzymes working on chemically different sugars may harness alternative agendas that reflect the chemistry of their substrate.

What then governs the specificity of a clan GH-A enzymes? We have previously commented[4,12] that the interactions around C1 and O2 of enzymes acting on gluco- (including xylose) and manno-configured substrates are extremely similar. This is again the case here. Both GH26 gluco- and manno-specific enzymes display near identical geometry for the catalytic acid, nucleophile, and the O2-interaction residue (histidine in GH26 enzymes, although it is normally asparagine in clan GH-A [7]), see Figure 2 A. The conservation of the O2-interaction geometry strongly sug-

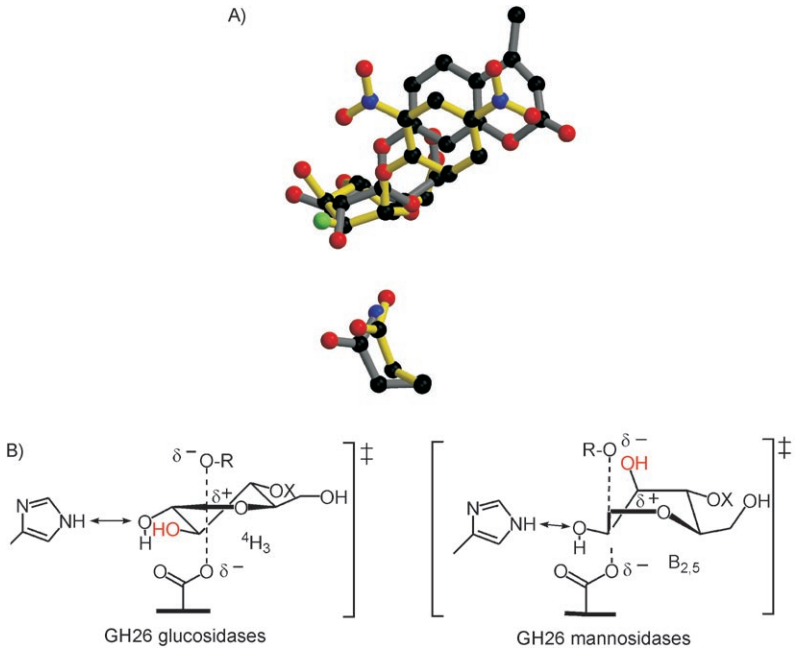


Figure 2. A) Overlap of the "Michaelis" complexes for manno- and gluco-specific enzymes from family GH26 (*Ct*Lic26A, gray; *Cj*Man26A, yellow). B) Comparison of (predicted) $^{4}H_{3}$ and $B_{2,5}$ transition states for glucoside and mannoside hydrolysis by clan GH-A enzymes (O3, discussed in the text below, is indicated in red as a reference).

gests that O2 lies pseudoequatorial during the hydrolysis of both glucosides and mannosides, again consistent both with $^4H_3$ and $B_{2,5}$ conformations for their respective transition states (Figure 2B) and with the potential for interaction of O2 with the catalytic nucleophile.

The comparison of GH26 gluco- and manno-specific enzymes provides support for an earlier hypothesis that the interactions and environment of O3 contribute to the specificity and reaction coordinate. O3 is the atom whose position differs significantly between $^4H_3$ and $B_{2,5}$ transition states in which O3 lies equatorial in $^4H_3$, but pseudoaxial (and "up") in the latter. Comparison of glucosidases and mannosidases suggested that the O3 interacting residues of these two classes lie in different positions. The requirement for direct hydrogen-bonding groups tethering O3 is less important in the case of *Ct*Lic26A, as O3 is restrained to an equatorial position by virtue of the β-1,3 glycosidic linkage to the adjacent sugar.

The conformation of the reaction transition state is crucial if one is to harness the extremely tight binding of the transition state (resulting from the amazing catalytic proficiency of these enzymes) for specific enzyme inhibition in a therapeutic context. In this light, the inhibition of Golgi α-mannosidases is emerging as a potential anti-cancer target.[21–23] Mannosidase II action is a prerequisite for the action of Golgi β-1,6-*N*-acetylglucosaminyltransferase V (MGAT5). This latter enzyme plays a key role in malignancy and cancer progression, with MGAT5-deficient mice exhibiting suppressed tumor growth and metastasis.[24] The evidence strongly points to retaining α-mannosidases that display the same, but reversed, conformational pathway as the family GH26 retaining β-mannanases. This leads to the possibility of specific, targeted inhibition of clinically relevant enzymes. In this context, a large number of mannosidase inhibitors have been proposed in recent times with amidines,[25] pyridines[26] and isoquinuclidines,[27] and functionalized mannostatin and aminocyclopentitetrol analogues[21] that are notable for their binding to mannosidases.

Herein we have shown that related family GH26 enzymes acting on gluco-configured substrates offer an alternative catalytic pathway, with the flanking snapshots of a Michaelis complex and intermediate suggesting catalysis progress through a $^4H_3$ (or related ^4E) transition state. This work contributes to the evolving landscape of conformational pathways and provides further incentive to develop powerful and specific transition-state mimics as drug candidates for the future. General rules are certainly beginning to emerge. Whether an enzyme is "*exo*" or "*endo*" in activity may also play a significant role. One would predict that *exo*-acting enzymes, in which the glycon sugar is not tethered to an extended oligo- or polysaccharide chain, are able to explore a larger conformational space than *endo*-acting enzymes whose −1 subsite glycon is necessarily restrained by the oligosaccharide chain to which it is linked. The chemistry of the sugar, although important, does not dominate: there is strong evidence for both $B_{2,5}$ and $^3H_4/^3E$ transition states for mannoside hydrolysis and both $^4H_3$ and $^{2,5}B$ for the hydrolysis of gluco-configured substrates. Similarly, the evolutionary origin and structure of the enzyme is not overriding; only subtle changes in the enzyme are observed for GH-A clan enzymes in order for them to favor different conformational pathways for the hydrolysis of mannosides or glucosides.

Experimental Section

Compound **1** was purchased from Sigma-Aldrich. The synthesis of compound **2** will be described shortly. E109A *Ct*Lic26A was prepared, according to reference [5], in the non-His-tagged form that is suitable for ligand-binding studies. Amino acid substitution of the catalytic nucleophile E222 of *Ct*Lic26A to Gln (E222Q) was generated by the QuickChange site-directed mutatgenesis kit (Stratagene) by using pCF1 as template DNA and primers 5′ CCCATCATTATAGCACAGTTTGCATCAGCTGAAATAGGCG 3′ and 5′ CGCCTATTTCAGCTGATGCAAACTGTGCTATAATGATGGG 3′. The plasmid pCF1 (Taylor et al., 2005), which is a recombinant of pET21a, encodes protein *Ct*Lic26As, which does not contain a His tag. *E. coli* strain BL21 (DE3), harboring the recombinant plasmid, was cultured in Luria—Berton (LB) containing ampicillin (50 μg mL^{-1}) at 37 °C to an A_{600} of 0.6, and expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1.0 mM and incubated for an additional 5 h at 37 °C. *Ct*Lic26A E222Q was purified from material released from osmotic shock by anion-exchange chromatography followed by size-exclusion chromatography.

E109A *Ct*Lic26A was preincubated with powdered **2** and crystallized in ammonium sulfate (0.15 M), polyethylene glycol (PEG) 5 K monomethyl ether (30%; w/v) buffered to pH 6.5 with 100 mM 2-(4-morpholinyl)ethanesulfonic acid (MES). Crystals of E222Q *Ct*Lic26A were obtained in a similar manner with the inclusion of **1** instead of **2**. X-ray crystal data for E109A *Ct*Lic26A with **2** were collected on beamline ID14-4 of the European Synchrotron Radiation Facility (ESRF) to 1.4 Å resolution. Data were processed with MOSFLM from the CCP4[28] suite. The data are 99.8% (98.9) complete with an R_{merge} of 0.053 (0.17), a mean $I/\sigma I$ of 19.3 (8.0), and multiplicity of observation of 4.8 (3.9) (outer resolution shell data in parentheses). For E222Q *Ct*Lic26A with **1** the X-ray diffraction data were collected on ID14-4 at the ESRF and are 99.9% complete with an R_{merge} of 0.047 (0.242), a mean $I/\sigma I$ of 22 (4.3), and a multiplicity of 4.8 (3.5). Both data sets show very low overall B values reflecting well-ordered crystals. The structures were refined with REFMAC[29] (E109A with **2**, final R_{cryst}/R_{free} of 0.15/0.18; E222Q with **1**, final R_{cryst}/R_{free} of 0.15/0.18). Structural figures were drawn with BOBSCRIPT.[30]

A full table of X-ray crystal data and structure quality data is provided in the Supporting Information.

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