Unlocking the Hydrolytic Mechanism of GH92 α-1,2-Mannosidases: Computation Inspires the use of C-Glycosides as Michaelis Complex Mimics

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Abstract: The conformational changes in a sugar moiety along the hydrolytic pathway are key to understand the mechanism of glycoside hydrolases (GHs) and to design new inhibitors. The two predominant itineraries for mannosidases go via $S_1\rightarrow B_{1,2} \rightarrow S_2$ and $S_1 \rightarrow H_4 \rightarrow C_4$. For the CAZy family 92, the conformational itinerary was unknown. Published complexes of Bacteroides thetaiotaomicron GH92 catalyst with a $S_2$-glycoside and mannoimidazole indicate a $C_4 \rightarrow H_4 / 1 S_1 \rightarrow 1 S_2$ mechanism. However, as observed with the GH125 family, $S$-glycosides may not act always as good mimics of GH's natural substrate. Here we present a cooperative study between computations and experiments where our results predict the $E_1 \rightarrow B_{1,2} \rightarrow S_2 \rightarrow 1 S_2$ pathway for GH92 enzymes. Furthermore, we demonstrate the Michaelis complex mimicry of a new kind of C-disaccharides, whose biochemical applicability was still a chimera.

Introduction

The regulation of N-glycan modifications is crucial to maintain protein quality control and the functional development of glycoproteins in the endoplasmic reticulum and the Golgi apparatus.[1] Glycoside hydrolases (GHs) are biocatalysts dedicated to cleave glycosidic bonds connecting individual monosaccharides. Several families of GHs are also involved in N-glycan biosynthesis. Some of these enzymes that are involved in the protein folding and the function regulation are essential for the control of congenital disorders of glycosylation – also known as carbohydrate-deficient glycoprotein syndromes.[2,3] Among those catalysts, α-mannosidases are irreplaceable in the regulation of the mannose-containing N-glycan processing. Following the CAZy classification,[4] GH families 38,[5] 47,[6] 76,[7] 92,[8] 99[9] and 125[10] are responsible for N-glycan cleavage.

The inhibition of a GH relates to its enzymatic mechanism. Once the substrate (a saccharide) joins the active site of the hydrolase, the sugar in the $−1$ subsite ($−1$ sugar) undergoes a conformational change during hydrolysis. When the process overcomes the highest energy state, also known as the transition state (TS), the $−1$ sugar becomes an oxocarbenium ion-like moiety.[12,13] One of the strategies to inhibit a GH is based on designing TS mimics whose conformation is similar to the oxocarbenium ion-like ring.[14–16]

In parallel, developing chemical derivatives of the natural substrate of GHs has emerged as a procedure to inhibit glycosidases. The most common technique utilizes sulfuration of the glycosidic bond ($S$-glycoside).[17] In some experiments, the crystallization of GHs in complex with $S$-glycosides shed light on the conformation of the natural substrate in the Michaelis complex (MC, reactant).[18,19] However, their MC mimicry was not observed in GH125. The natural substrate-related $S$-glycoside predicted a non-distorted $−1$ sugar moiety, while computations and further experiments showed a distorted one.[20] Comparing the available GH125 complexes reveals ($S$-glycoside – PDB 3QT9,[10] and natural substrate – PDB 5M7P,[19] that the substitution of the glycosidic oxygen by sulfur leads to a lengthening of the glycosidic bond (from 1.45 Å to 1.87 Å) and a $C−C$ angular readjustment (from 108.4° to 103.7°). Due to the topology of the GH125 active site, this structural change resulted in a hydrogen bond rearrangement between the $−1$ sugar and the enzyme, and a consequent conformational change.

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Since we talk about the ring mimicry, the conformational study of 6-membered rings (6-rings) is the base to classify and quantify the enzymatic itineraries of GHs. In 1975, Cremer and Pople developed a mathematical expression based on puckering coordinate(s) allowing the graphical representation of the conformational space of a ring.\(^{[21]}\) As depicted in Figure 1, a Mercator representation projects the puckering space in a two-dimensional surface where all the conformations of a pyranose ring are presented. In this work, we will use the IUPAC notation of pyranose conformations.\(^{[22]}\) By the principle of least nuclear motion,\(^{[23]}\) the conformations of the reactants (MC) and products (PC) must surround the TS, following an ideal linear pathway. In α-mannosidases, we observe two main itineraries: \(^{3}S_{4} \rightarrow B_{2,3} \rightarrow S_{1}\) (GH38, 76, and 125; the expected pathway for GH92, Figure 1 – yellow arrow) and \(^{3}S_{1} \rightarrow H_{5} \rightarrow C_{4}\) (GH47; the alternative pathway, Figure 1 – blue arrow). Do the significant double-bond character between the pyranic oxygen and the anomeric carbon, the C2-C1-O5-C5 torsion angle tends to zero, and the conformational space of the TS mimics was recently found around a linear region separating the Mercator surface (Figure 1 – blue line).\(^{[15]}\)

In the present work we focus on the inverting Ca\(^{2+}\)-dependent exo-α-1,2-mannosidase, whose catalytic residues are a proton donor (Glu) and an assistant base (Asp). In Ref. [8], Zhu et al. characterized and crystallized Bacteroides thetaiotaomicron GH92 (BT3990, BtGH92) complex with α,1,2-5-mannobiose 2 (Figure 2) in the presence and in the absence of Ca\(^{2+}\) (MC mimic), and mannoimidazole 4 (Figure 2 – MVL) in the presence of Ca\(^{2+}\) (TS mimic). The MC mimic shows an undistorted \(-1\) sugar moiety (\(\alpha\)), both in the presence and in the absence of Ca\(^{2+}\). The TS mimic shows a \(\alpha\)\(\rightarrow\)\(\beta\)\(\rightarrow\)distorted conformation. Connecting both regions (Figure 1, green arrow), the experiments show an unexpected \(\alpha\)\(\rightarrow\)\(\beta\)\(\rightarrow\)\(\alpha\)\(\rightarrow\)\(\beta\)\(\rightarrow\)distorted conformation. Furthermore, analyzing the available MC mimic complexes (PDB 2WW1 and 2WW3), the catalytic water is not kept in a position allowing the nucleophilic attack (>4 Å), while the TS mimic (PDB 2WZS) shows a water molecule at a distance of ~3 Å from the anomeric center (Figure 3). This fact and the resulting pathway disrespects the least nuclear motion principle indicate a possible conformational mismatch between the S-glycoside 3 and the natural substrate 1 (as observed in GH125).

While the hydrolytic mechanism of GH92 enzymes remains locked, our goal is to decipher the conformational changes

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**Figure 1.** (top) Representation of different conformers of a 6-membered ring molecule (chair – C, boat – B, envelope – E and, half-chair – H) following the IUPAC notation.\(^{[25]}\) Mercator projection of the Cremer and Pople’s puckering coordinates’ sphere for a 6-membered ring. (bottom) –1 sugar moieties in PDB 2WW1 (red dots), 2WW3 (green dots) and 2WZS (purple triangles) are depicted over the Mercator conformational surface. The experiments in Ref. [8] describe the \(S_{2}\)\(\rightarrow\)\(H_{5}\)\(\rightarrow\)\(C_{4}\) conformational itinerary (green arrow), while the expected (yellow arrow) and alternative (blue arrow) pathways are \(S_{2}\)\(\rightarrow\)\(B_{2,3}\)\(\rightarrow\)\(S_{1}\) and \(S_{1}\)\(\rightarrow\)\(H_{5}\)\(\rightarrow\)\(C_{4}\) respectively. The blue line represents an analytical function from Ref. [15] connected to the TS mimic conformational space.

**Figure 2.** Schematic representation of the molecular structures of the substrates used to study the hydrolytic mechanism of GH92 α-mannosidases (each ring moiety occupies either the \(-1\) or the \(+1\) subsite as noted in this representation).

**Figure 3.** Schematic diagrams of BT3990 (BtGH92) complexed with α,1,2-5-mannobiose 3 and mannoimidazole 4.\(^{[8]}\)
occurring over the –1 sugar from the formation of the Michaelis complex to the delivery of the inverted product. Our most burning question in this work solved the structural differences between the S-glycoside 3 and MVL 4 complexes (Figure 3), and why is the catalytic water not kept close to the S-glycoside. The answer could be related to the abrupt conformational change of the –1 sugar. After the inspection of the structure, we observed the most relevant difference in the vicinity of the Ca$^{2+}$ cation. In the presence of MVL 4, the catalytic water is coordinated to the calcium cation. In the presence of S-glycoside 3, the hydroxyl group (OH) in carbon 2 (C2) is occupying the position of the catalytic water. With MVL 4, the hydroxyl at C2 interacts with the proton donor (E533).

Considering all aforementioned evidence, quantum-mechanics (QM) calculations could shed light on the conformational itinerary followed by the natural substrate in GH92 enzymes. Starting from the BtGH92-MVL crystal structure (PDB 2WZS), we constructed a full-QM cluster model of the active site. As a computational itinerary followed by the natural substrate in GH92 enzymes. Starting from the BtGH92-MVL crystal structure (PDB 2WZS), we constructed a full-QM cluster model of the active site. As a computational itinerary followed by the natural substrate in GH92 enzymes, we constructed a full-QM cluster model of the active site. As a computational itinerary followed by the natural substrate in GH92 enzymes, we constructed a full-QM cluster model of the active site. As a computational itinerary followed by the natural substrate in GH92 enzymes, we obtained the potential energy barriers of $\Delta E_1$ = 14.1 kcal · mol$^{-1}$ and $\Delta E_0$ = −2.7 kcal · mol$^{-1}$. This result is in good agreement with the available kinetics parameters.

Results and Discussion

The optimized cluster model (depicted in Figure 4 as MC) presents a distorted $E_1/B_{2,5}$ conformation for the –1 sugar ring. This result differs significantly from the $^{\text{4}C_1}$ conformation observed in the S-glycoside 3. In our scan calculation, the proton in E533 (equivalent to E494 of EfGH92) was transferred to the glycosidic oxygen, the cleavage of the glycosidic bond, and the nucleophilic attack of the catalytic water were activated by restraining the involved distances. The optimized structures of MC, TS ("TS-like") and PC are presented in Figure 4. The obtained potential energy barriers of $\Delta E_0$ = 14.1 kcal · mol$^{-1}$ and $\Delta E_1$ = −2.7 kcal · mol$^{-1}$. This result is in good agreement with the available kinetics parameters.

X-ray crystallography showed that WT and the mutant form a tetramer where ligands and Ca$^{2+}$ ions are allocated in each active site cavity (–1 and +1 subsites). On the one hand, three of the four C-glycosides 2 attached to WT exist in a conformation corresponding to the QM-optimized cluster model (E$E_1$/B$B_{2,5}$), while one outlier presents a $^{\text{3}S_1}$/^$B_5$ structure (Figure 4 – green dots). On the other hand, the natural substrate 1 attached to the mutant present one of the four structures in the $E_1$ region, two of them in the $E_4$/H$E_1$ region, and the last one in the $H_4$/S$S_1$ space (Figure 4 – purple dots). This evidence together with the conformations found in the BtGH92-MVL complex 4 indicate a simplified conformational itinerary crossing the $E_5$/$S_1$ region (Figure 4 – green arrow).

Following the –1 sugar conformation along the reaction pathway (Figure 4 – red dots and black dashed line), we can conclude that the itinerary in our cluster model is $E_1/B_{2,5}$ + $B_{2,5}$, $E_1$ + $S_1/B_{3,5}$, being aware of the subtle conformational changes observed in the –1 sugar both experimentally and computationally, and the dynamic nature of the biochemical systems, the accuracy of results is limited. The experimental results lead to an MC in the vicinity of $E_1$ and a TS crossing $H_4$/S$S_1$. While the calculations suggest an MC in $E_1/B_{3,5}$ and a TS in $B_2/E_5$. In any case, investigated changes are only minor. Nevertheless, when we slightly simplify the nomenclature, we can conclude that GH92 α-1,2-mannosidase follows an $E_1$ – $B_{2,5}$, $S_1$ – $E_5$ conformational itinerary (Figure 4, middle).

The analysis of crystal structures of active sites revealed (Figure 5) that the distorted α-D-mannopyranosyl moiety occupies the –1 subsite, while the +1 subsite is occupied by an undistorted 2-CH$_2$- and 2-O-mannosyl leaving group. Comparing the EfGH92-C-glycoside complex and the PDB 2W3 (thioglycoside) structure, we observe that the hydroxyl group at C2 of the S-glycoside occupies the same position as the catalytic water in the C-disaccharide complex. Due to the hydrophobic nature of the pseudo-glycosidic methylene group, the E494 catalytic acid residue changes its orientation, and a
water molecule interacts with the OH group at C2 of the C-glycoside. The closest water to the anomeric carbon is further than 4 Å in case of the S-glycoside, while a well-oriented catalytic water is coordinated to Ca$^{2+}$ at ~3 Å from the anomeric center of the C-glycoside (Figure 5 – C).

In Figure 5 – D, we also depict the main hydrogen bond interactions present in the E494Q EfGH92-mannobiose complex. As observed in the QM-optimized BtGH92 cluster model, the OH group at C2 interacts with the oxygen of the mutated glutamine residue Q494. The NH$_2$ group of the mutated residue interacts with the glycosidic oxygen, properly oriented for a hypothetic proton transfer. The hydroxyls at C3 and C4 interact with aspartic acid D313. The catalytic water interacts with two aspartic acid residues, D602 and D604. Finally, the hydroxymethyl arm interacts with a water molecule and serine S66. These observations are in good agreement with the interactions observed in our calculations, strengthening the viability of the model.

**Conclusion**

In conclusion, computations in this study have newly been a powerful tool to decrypt the reaction mechanism of an enzyme-substrate biosystem. Both experimental results and calculations confirm that the catalytic mechanism of GH92 α-1,2-mannosidases follows the $E_1\rightarrow B_{2,5}/S_2\rightarrow E_1$ itinerary (Figure 4). As observed in GH125 enzymes, S-glycosides may not act as good MC mimics, due to different interaction patterns with the active site residues, and, in this case, also, with a metal cation. The
conformational difference between the C-glycoside and the S-glycoside allows the approach of the catalytic water to the anomeric carbon, in the first case, where the water is coordinated with the Ca$^{2+}$ cation. We identify a new class of MC mimic through the substitution of glycosidic oxygen by the CH$_3$ group. This chemical change keeps the conformation of the sugar, but the glycosidic position becomes hydrophobic, suppressing thus the interaction with the catalytic residue.

**Experimental Section**

Protein production and crystallography procedures, details about the synthesis and conformational study of the C-analogue of α-1,2-mannobiose 2 and computational details are available in the Supplementing Information.

**Structural data availability**

The atomic coordinates and structure factors have been deposited in the Worldwide Protein Data Bank (http://wwpdb.org/) under access code 7FE1 and 7FE2.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available in the supplementary material of this article.

**Keywords:** carbohydrates · conformations · enzymology · inhibitors · quantum mechanics

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