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Glycosidase mechanisms: Sugar conformations and reactivity in endo- and exo-acting enzymes

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Abstract

The enzymatic breakdown of carbohydrates plays a critical role in several biological events and enables the development of sustainable processes to obtain bioproducts and biofuels. In this scenario, the design of efficient inhibitors for glycosidases that can act as drug targets and the engineering of carbohydrate-active enzymes with tailored catalytic properties is of remarkable importance. To guide rational approaches, it is necessary to elucidate enzyme molecular mechanisms, in particular understanding how the microenvironment modulates the conformational space explored by the substrate. Computer simulations, especially those based on *ab initio* methods, have provided a suitable atomic description of carbohydrate conformations and catalytic reactions in several glycosidase families. In this review, we will focus on how the active-site topology (pocket or cleft) and mode of cleavage (endo or exo) can affect the catalytic mechanisms adopted by glycosidases, in particular the substrate conformations along the reaction coordinate.

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Abbreviations

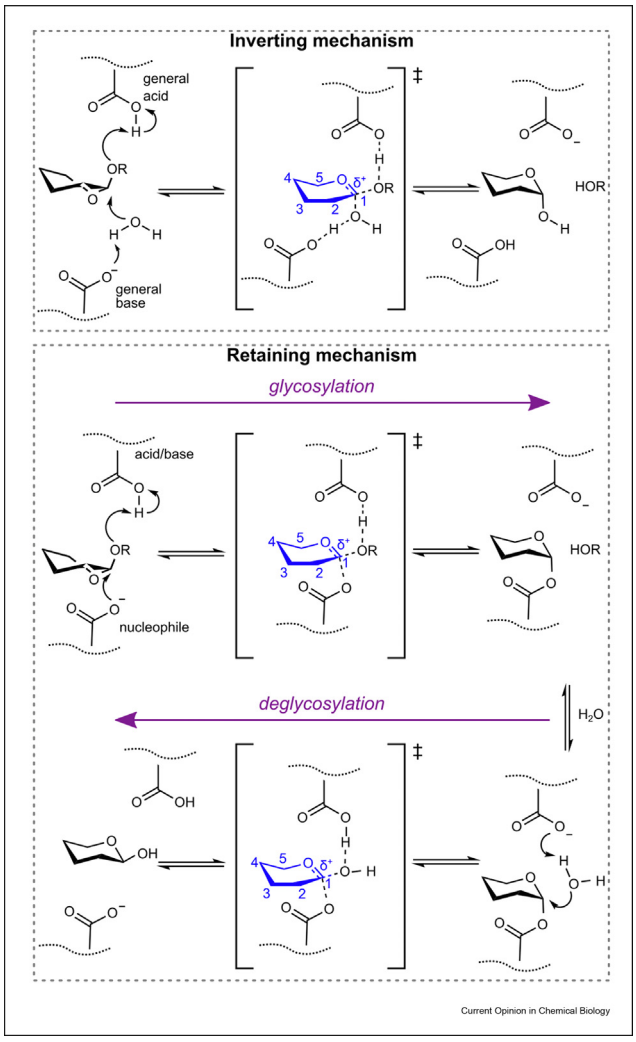
GH(s), glycoside hydrolase(s); TS, transition state(s); CAZy, carbohydrate-active enzyme; PDB, protein data bank; MD, molecular dynamics; MC, Michaelis complex; FEL, free energy landscape; GEI, glycosyl-enzyme intermediate; QM/MM, quantum mechanics/molecular mechanics.

Introduction

Glycosidases (or glycoside hydrolases, GHs) are the main catalysts for the cleavage and modification of carbohydrates. They are currently grouped in approximately 170 families, according to the carbohydrate-active enzymes (CAZy) database [1]. The vast majority of GHs share the same catalytic mechanism: acid/base catalysis with retention or inversion of the anomeric configuration [2,3]. The reaction is assisted by two essential residues: a proton donor and a nucleophile (or general base), usually glutamate or aspartate residues. Inverting enzymes operate by a single nucleophilic substitution, while retaining GHs follow a double displacement mechanism via formation and subsequent hydrolysis of a covalent glycosyl-enzyme intermediate (glycosylation and deglycosylation steps as shown in Figure 1). Both types of reactions involve oxocarbenium ion-like transition states (TS), which are high-energy metastable states in which covalent bonds involving the catalytic residues are partially broken or formed [4] (Figure 1). Variations on the classical inverting or retaining mechanisms have been described. Some GH families use substrate-assisted catalysis, where the nucleophile residue is absent and the TS is reached through the mediation of a substrate substituent, either a *N*-acetyl or a hydroxyl group [5–7]. Moreover, some GHs have alternative nucleophiles [8–10] or acid/base [11], or rely on cofactors to perform the hydrolysis [12,13]. The conformations adopted by the substrate along the reaction coordinate, in particular that of the TS, are relevant for the rational design of inhibitors and activity-based probes for GHs. Thus, significant effort has been devoted in recent years to uncover GH catalytic itineraries.

GH active sites are fine-tuned to cope with the stereochemical complexity of their substrates and stabilize

Figure 1



Classical mechanisms of enzymatic glycosidic bond hydrolysis catalyzed by inverting (upper panel) or retaining (lower panel) GHs. Pyranose carbon atoms in the transition states (blue) are numbered.

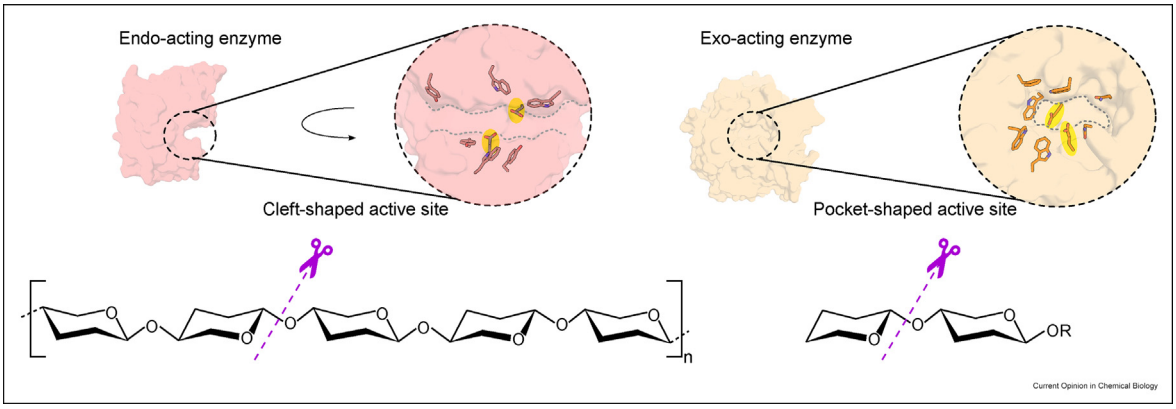
the TS state configuration. They can be classified into endo or exo, according to the cleavage pattern produced (Figure 2). While endo-acting GHs (hereafter endo-GHs) cleave the middle of a carbohydrate chain, exo-acting GHs (hereafter exo-GHs) recognize terminal sugars, in general the non-reducing end. The difference between these two modes of operation is reflected by the distinct active-site topologies, especially adapted to the mode of action and to cope with different substrate lengths [14]. Endo-GHs often cleave polymeric carbohydrate chains and display an open and cleft-shaped active site, releasing products with different degrees of oligomerization [14]. They might also exhibit processive hydrolysis, where the release of the product occurs while the enzyme remains bound to the remaining chain of the substrate. Processive GHs often have the catalytic cleft modified as tunnel-shaped active site [14]. On the contrary, exo-GHs generally cleave di- or oligosaccharides that bind to a pocked-shaped active site [14], releasing a specific product (Figure 2), or the terminal sugar of large glycolipids and glycoproteins. They have also been found displaying a deep pocket, in a type of substrate and product-assisted catalysis [15,16]. The active-site pocket of exo-GHs can also be an extended one, in which residues at the surface of the enzyme can bind the partially solvent-exposed substrate. In such cases, the exo-GHs are able to bind to polymeric and/or oligomeric substrates [17–19]. In this review, we will focus on the endo/exo activities in enzymes that exhibit open clefts or pocket active sites.

On the reactive sugar conformation and its role for catalysis

The oxocarbenium ion-like TS of the reactions catalyzed by GHs is stabilized through the delocalization of the pyranic oxygen lone pairs, which is optimally achieved when the atoms C2, C1, O5, and C5 are coplanar (Figure 1). This restricts the number of conformations that can be adopted by the reactive sugar at the TS ($^4H_3$, $^3H_4$, E_3, ^3E, E_4, ^4E, $B_{2,5}$ $^{2,5}B$, or slightly distorted variants), which is characterized by significant distortion in the pyranose ring [20–22]. Distortions that are "on the pathway" toward the TS also occur at the Michaelis complex (MC) of GHs [23,24], especially for β-GHs and several α-GH families. It has been found that these distortions not only place the glycosidic bond in an axial orientation but also facilitate the reaction by increasing the anomeric charge and elongating the glycosidic bond [25,26]. Some α-GHs, such as GH13 α-amylase, do not gain by distorting the substrate, since the leaving group is already axially oriented at the MC. Thus, in this case, no distortion of the reactive sugar at the Michaelis complex is observed in experiments or simulations [27,28]. The sequence of conformations adopted by the reactive sugar along the catalysis reaction coordinate delineates the *conformational catalytic itinerary*, in which the substrate distorts upon evolving from the MC to the TS and adopts a different conformation once the reaction intermediate (GEI for classical retaining GHs, oxazolinium ion-type or epoxide-type intermediate for retaining GHs operating via substrate-assisted catalysis) or the product (for inverting GHs) is formed. Determining the conformational catalytic itineraries has been instrumental for the design of selective inhibitors and activity-based probes for GHs [29].

The conformation of carbohydrates in complex with enzymes can be retrieved from released structures available on the protein data bank (PDB) database [30]. However, the strong dependence on data resolution, the usage of

Figure 2



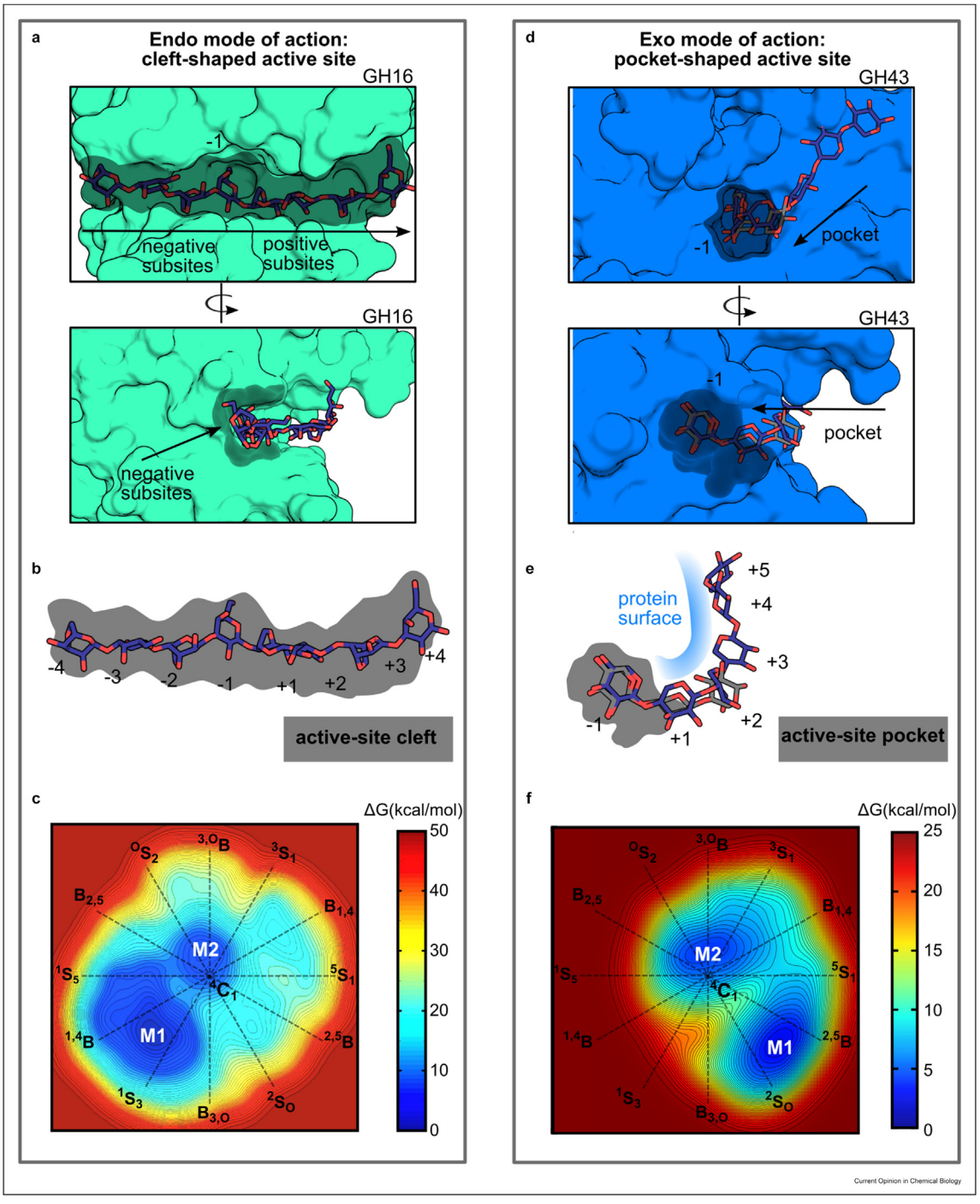
Endo and exo-modes of action of GHs. Endo-acting enzymes harbor a cleft-shaped active site that binds to the middle of a polysaccharide chain. Exo-acting enzymes harbor a pocket-shaped active site that recognizes oligosaccharides released by endo-GHs (or from alternative sources) in the extremity (most commonly the non-reducing end [14]). Structures represented correspond to an endo-glucanase (PDB 1U0A [49]) and β-glucosidase (PDB 3VIF, [50]). Catalytic residues are highlighted by yellow circles (mutations in PDB 1U0A were reverted for representation).

non-catalytic states for crystallization of the enzyme-substrate complexes, and/or the limitations of currently available classical force fields can impair the reliability of the computational models [31]. To overcome these limitations, computer simulations using well-parametrized force fields or semi-empirical methods have provided insightful results [10,32]. However, the usage of density functional theory (DFT)-based methods to obtain the conformational landscapes of sugars has been necessary, since often the puckering coordinates are not accurately reproduced by molecular mechanics force fields or semi-empirical approaches, which leads to an over exploration of the canonical chair or even non-catalytically relevant conformations [17,33–36].

Several theoretical studies (recently reviewed in [37]), especially using quantum mechanics/molecular mechanics (QM/MM) simulations, have investigated conformations of the reactive sugars in enzyme active sites and how these relate to conformational catalytic itineraries, most of times matching with available experimental structures (reviewed by [24,38,39]). One example is the investigation of the conformational catalytic itinerary of the inverting GH134 endo-β-mannanase [40]. The unexpected inverted chair ($^{1}C_4$) conformation observed in the crystal structures of both the Michaelis complex (MC) (using an inactive mutant of the acid/base residue) and the product complex, indicated a conformational catalytic itinerary in the southern hemisphere of the Cremer–Pople sphere, while previous β-mannanases have been shown to follow the northern $^{1}S_5 \rightarrow [B_{2,5}]^{\ddagger} \rightarrow {}^{O}S_2$ catalytic itinerary. QM/MM metadynamics simulations confirmed a novel "southern" $^{1}C_4 \rightarrow [^{3}H_4]^{\ddagger} \rightarrow {}^{3}S_1$ catalytic itinerary. Moreover, the simulations captured the subsequent relaxation of the reactive sugar from $^{3}S_1$ toward a $^{1}C_4$ at the reaction products, providing the missing connection between the two snapshots of reactants and products that had been obtained by X-ray crystallography.

One intriguing question of GH catalysis is to find the determinants of substrate distortion across GH families. Several theoretical studies have demonstrated that not only the active-site architecture but also intrinsic properties of the substrate play a role in shaping the substrate distortion upon binding. In particular, it has been found that the substrate conformation observed in the enzyme corresponds to a minimum energy conformer in the conformational free energy landscape (FEL) of the free sugar [41,42]. For instance, even though the conformational FEL of free β-glucose points toward a more stable conformation around the relaxed $^{4}C_1$ [41], there is a local minimum energy located around the distorted skew-boats that displays favorable structural/electronic properties for the enzyme to exploit in the catalysis reaction (the C1-O bond is long and the anomeric charge is high, compared with the same properties in the chair conformations). Interestingly, the most favored substrate conformation in enzyme is close to a local minimum energy of the free substrate [25]. Similarly, the conformational FELs for isolated β-xylose and β-mannose display the lowest minimum close to chair conformation, but secondary distorted low energy minima correspond to conformations observed in productive Michaelis (enzyme-substrate) complexes [42,43]. Simulations with other levels of theory and considering other sugars such as β-GlcNAc later showed similar relationships [44]. When comparing conformational landscapes of sugars in enzymes with the ones of isolated sugars, it becomes clear that GHs do not select distorted conformations of their substrates randomly, but capitalize on the intrinsic properties of the free substrate.

Figure 3



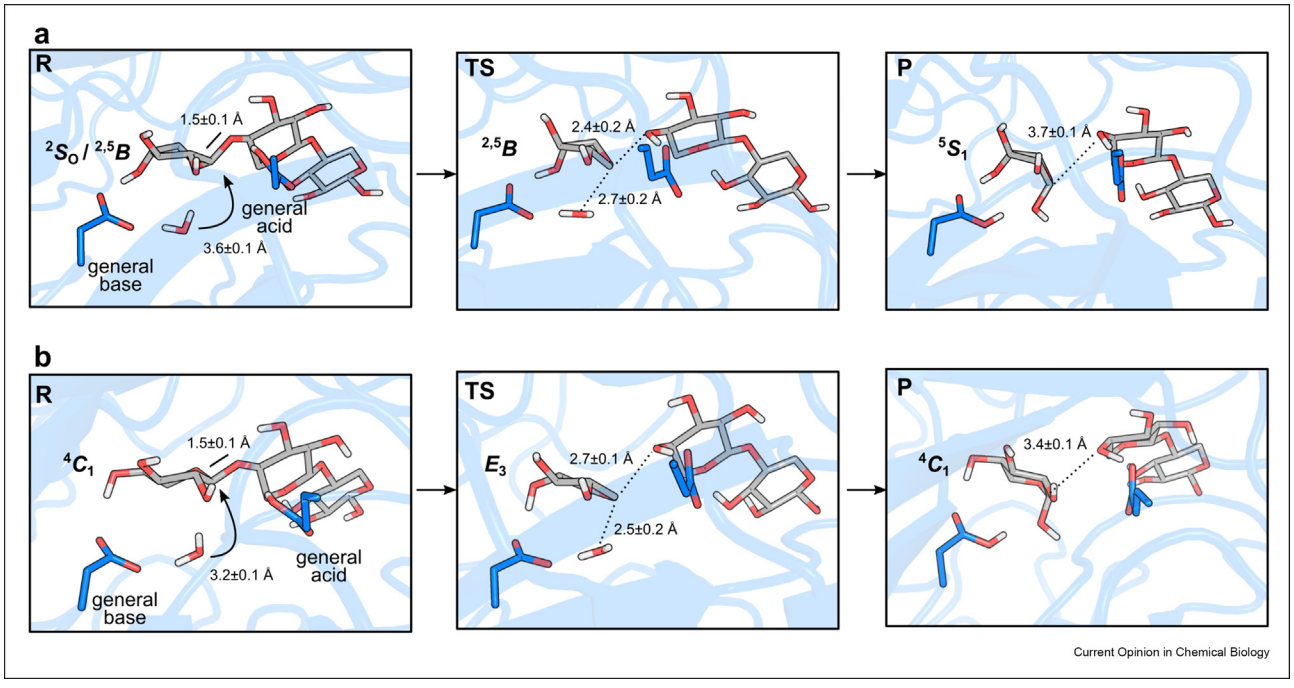
Comparison between endo and exo-GH active sites and their influence in substrate binding and distortion. (**a**) Structure of a representative family 16 endo-GH (endo-agarase in complex with octasaccharide (PDB 4ATF, [51])), highlighting the extended active-site cleft (grey) and the productive negative and positive subsites. (**b**) Schematic representation of the tightly confined substrate bound to the active-site cleft of an endo-GH. (**c**) Computed free energy landscape (FEL), with respect to ring distortion of β-glucopyranosyl at the *-1* subsite of GH16 β-endoglucanase displaying one major minimum (M1) and a secondary minimum (M2) [25,39], with 6 kcal/mol of difference. (**d**) Structure of an GH43 exo-oligoxylanase/β-xylosidase in complex with xylotriose (grey sticks) (PDB 6XN2, [17]) and with docked xylohexaose (dark blue sticks), highlighting the active-site pocket (grey). (**e**) Schematic representation of the substrate bound to family 43 exo-GH, in which the *-1* subsite is buried into the pocket and the leaving group is at the protein surface. (**f**) Computed FEL of β-xylosyl at the *−1* subsite of GH43 exo-oligoxylanase/β-xylosidase displaying two iso-energetic minima (~1 kcal difference between M1 and M2) [17]. Subsites nomenclature was based on the proposed for GHs [52]. FELs of (c) and (f) are contoured at 0.5 kcal/mol. Image (c) has been reprinted (adapted) with permission from [25]. Copyright (2011) The American Chemical Society. Image (f) has been adapted from reference [17].

Alternative itineraries: some exo-acting GHs do not distort their substrates

The observation that GHs favor the distorted conformation of their substrates before catalysis, positioning the scissile bond in a pseudo-axial orientation suggests that undistorted substrate conformations are highly energetic and/or unreactive in these GHs [24]. In fact, GH16 endo-β-glucanase favors the distortion into a $^{1}S_{3}/^{1,4}B$ conformation (hereafter $\sim^{1}S_{3}$) of the reactive glucosyl that is approximately 6 kcal/mol more stable than the fully relaxed $^{4}C_{1}$ conformation (considering the enzyme-substrate complex) [25] (see scheme in Figure 3 a-c). The distorted conformation is stabilized by sugar-enzyme interactions at the *-1* enzyme subsite (mainly, the strong 2-OH$\cdots$ nucleophile interaction), as well as the steric constraints on the positive subsites that favor an axial orientation of the leaving group. The $\sim^{1}S_{3}$ conformation was also trapped in enzymes complexed with β-galactoside substrates [30,32,45], indicating that this conformation is relevant for the catalytic itinerary adopted by galactosidases. The xylanolytic enzyme GH11 endo-β-xylanase was found to exhibit two almost isoenergetic minima in the reactive sugar conformational FEL (difference of approximately 2 kcal/mol). However, when simulating the glycosidic bond cleavage, the energy barrier was much higher for the reaction starting from a non-distorted conformation in comparison with the one starting from $^{2}S_{O}$ (difference of more than 40 kcal/mol) (L Raich, PhD thesis, University of Barcelona, 2018). This indicates that the reaction starting from a relaxed $^{4}C_{1}$ conformation is not feasible for endo-β-GHs.

Recent studies have shown that the conformational catalytic itineraries of exo-GHs can differ from those of their endo counterparts. The crystal structure of the GH59 exo-β-galactocerebrosidase (GALC) in complex with a substrate analog, *p*-nitrophenyl-β-galactoside (Gal-β-pNP), reported that the reactive sugar is a relaxed $^{4}C_{1}$ conformation [46]. However, this conformation was not considered pre-activated for catalysis for a β-GH. In addition, docking and MD simulations of exo-galactosidases pointed to the existence of several interchanging conformers of the galactosyl substrate [32]. To tackle this intriguing data, the conformational FEL of the reactive sugar in GALC active site was calculated by QM/MM metadynamics [33]. The calculations revealed two local minima, one corresponding to the observed $^{4}C_{1}$, as well as another one with significant ring distortion, $^{1}S_{3}$ [25]. Further simulations of the catalytic reaction, starting from both conformations, revealed that GALC was able to operate through two different conformational itineraries, either starting from a distorted conformation ($^{1}S_{3} \rightarrow [^{4}H_{3}]^{\ddagger} \rightarrow {}^{4}C_{1}$)

Figure 4



Catalytic itineraries adopted by XacGH43_1. Representative structures of the reactant (R) transition state (TS) and product (P) states of XacGH43_1 catalytic itineraries (a) $^{2}S_{O}/^{2,5}B \rightarrow [^{2,5}B]^{\ddagger} \rightarrow {}^{5}S_{1}$ and (b) $^{4}C_{1} \rightarrow [E_{3}]^{\ddagger} \rightarrow {}^{4}C_{1}$, taken from QM/MM metadynamics simulations (adapted from [17]).

or starting from a relaxed one ($^4C_1 \rightarrow [^4H_3]^{\ddagger} \rightarrow {}^4C_1$) [33], with the first being slightly favored. This indicates that some exo-GHs display a different behavior with respect to their endo counterparts, for which the reaction can only take place from a distorted conformation.

An extensive and multidisciplinary characterization was recently reported for a xylanolytic enzyme, the GH43 exo-β-oligoxylanase (XacGH43_1). Unlike the classical exo-GHs pocket of GALC, in which the leaving group is totally exposed to the solvent, the XacGH43_1 active site consists of a modified "extended pocket," with possible additional positive subsites that allow the exo-oligoxylanase activity [17] (it is also noteworthy to highlight that the XacGH43_1 active-site architecture differs significantly from the typical cleft of endo-GHs). In XacGH43_1, positive subsites (beyond the *+1*) are located in the surface and are exposed to the solvent (Figure 3d–e). Both crystallographic structure and QM/MM metadynamics simulations on XacGH43_1 revealed two distinct substrate conformations at the Michaelis complex, a distorted ^2S_O conformation and a relaxed $^4C_1$ conformation [17] (Figure 3d–f). The ^2S_O conformation had been already observed in the active site of other xylanolytic enzymes that presumably follow a $^2S_O/^{2,5}B \rightarrow [^{2,5}B]^{\ddagger} \rightarrow {}^5S_1$ conformational itinerary [47]. The relaxed $^4C_1$ conformation instead was new, and thus it was indicative of an alternative itinerary (Figure 3f). The existence of two conformations for a β-GH is unusual; one would expect the $^4C_1$ conformation, in which the leaving group is equatorial, to be unreactive. However, QM/MM metadynamics simulations of the reaction coordinate evidenced that, similarly to GALC, it can be co-opted due to the flexibility of the leaving group, something that would be more difficult for an endo-acting GH. Therefore, two conformational catalytic itineraries, with distinct TS conformations, are operative for XacGH43_1: $^4C_1 \rightarrow [E_3]^{\ddagger} \rightarrow {}^4C_1$ and $^2S_O/^{2,5}B \rightarrow [^{2,5}B]^{\ddagger} \rightarrow {}^5S_1$ [17] (Figure 4). The versatility of exo-GHs to operate via different reaction pathways was also described for the bifunctional GH1 Os7BGlu26, which adopts the itinerary $^1S_3 \rightarrow [^4E/^4H_3]^{\ddagger} \rightarrow {}^4C_1$ for β-glucosides and $^1S_5 \rightarrow [B_{2,5}]^{\ddagger} \rightarrow {}^OS_2$ for β-mannosides [48].

Conclusions and perspectives

Computational chemistry has been crucial to sample all conformational states during GH-catalyzed reactions, including high energy but important states such as the TS, while structural biology methods (*e.g.* X-ray crystallography) usually only allow observation of a single stable state. Therefore, computational chemistry provides a richer understanding of conformation and reactivity, but benefits from being grounded by the experimental determination of states. In this review, we have focused on *conformational catalytic itineraries* of endo and exo-GHs, specifically on how the active-site topology can affect the reactive sugar conformation during catalysis. In the case of endo-GHs, only conformations in which the glycosidic bond is placed in an axial orientation can enable the substrate to reach the TS conformation. These distorted conformations lead to an increase in the anomeric charge and elongation of the glycosidic bond. Exo-acting GHs have been less studied in comparison. However, new data suggest that the presence of a pre-activated complex in chair conformation is a feature of some exo-GHs, leading to alternative itineraries that start from a relaxed $^4C_1$ conformation (such as $^4C_1 \rightarrow [^4H_3]^{\ddagger} \rightarrow {}^4C_1$ for GALC and $^4C_1 \rightarrow [E_3]^{\ddagger} \rightarrow {}^4C_1$ for XacGH43_1). This scenario is possible due to the lack of steric determinants at enzyme positive subsites, as exo-GHs have leaving groups that are totally or partially exposed to solvent. This makes it easier for the substrate to be driven to a suitable TS conformation even when the leaving group is in an equatorial orientation. This particular case—catalysis via alternative itineraries—probably holds for several other exo-GHs (either two itineraries or one itinerary starting from a non-distorted conformation), such as the recently investigated GH3 exo-glucosidases [15]. That GH43 and GH59 operate through the inverting and retaining mechanism, respectively, indicates that the viability of a catalytic itinerary starting from an undistorted conformation has no dependence of the stereochemical outcome. Deciphering the catalytic mechanism of other exo-GHs of biomedical and biotechnological relevance will assess the generality of this scenario. These findings will impact the strategies for the design of inhibitors and probes for exo-GHs. Having two (or more) TS conformations on the same enzyme provides an opportunity to target only one of them, avoiding cross-selectivity with related enzymes acting on the same substrate.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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