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Modeling catalytic reaction mechanisms in glycoside hydrolases

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Abstract

Modeling catalysis in carbohydrate-active enzymes is a daunting challenge because of the high flexibility and diversity of both enzymes and carbohydrates. Glycoside hydrolases (GHs) are an illustrative example, where conformational changes and subtle interactions have been shown to be critical for catalysis. GHs have pivotal roles in industry (e.g. biofuel or detergent production) and biomedicine (e.g. targets for cancer and diabetes), and thus, a huge effort is devoted to unveil their molecular mechanisms. Besides experimental techniques, computational methods have served to provide an in-depth understanding of GH mechanisms, capturing complex reaction coordinates and the conformational itineraries that substrates follow during the whole catalytic pathway, providing a framework that ultimately may assist the engineering of these enzymes and the design of new inhibitors.

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Glycosidases, Enzyme mechanisms, Sugar conformation, Quantum mechanics/molecular mechanics, Molecular dynamics, Metadynamics.

Introduction

Carbohydrate-active enzymes (CAZymes), which degrade, form, and modify carbohydrates and glycoconjugates, are of great interest because of the many roles that carbohydrates play in nature. These enzymes have a myriad of biotechnological and industrial applications, from biomass degradation to chemoenzymatic synthesis, as summarized by André et al. [1]. Here, we focus on glycoside hydrolases (GHs), which catalyze the hydrolysis of glycosidic linkages in glycosides, and we highlight recent contributions of molecular modeling to unravel their catalytic mysteries.

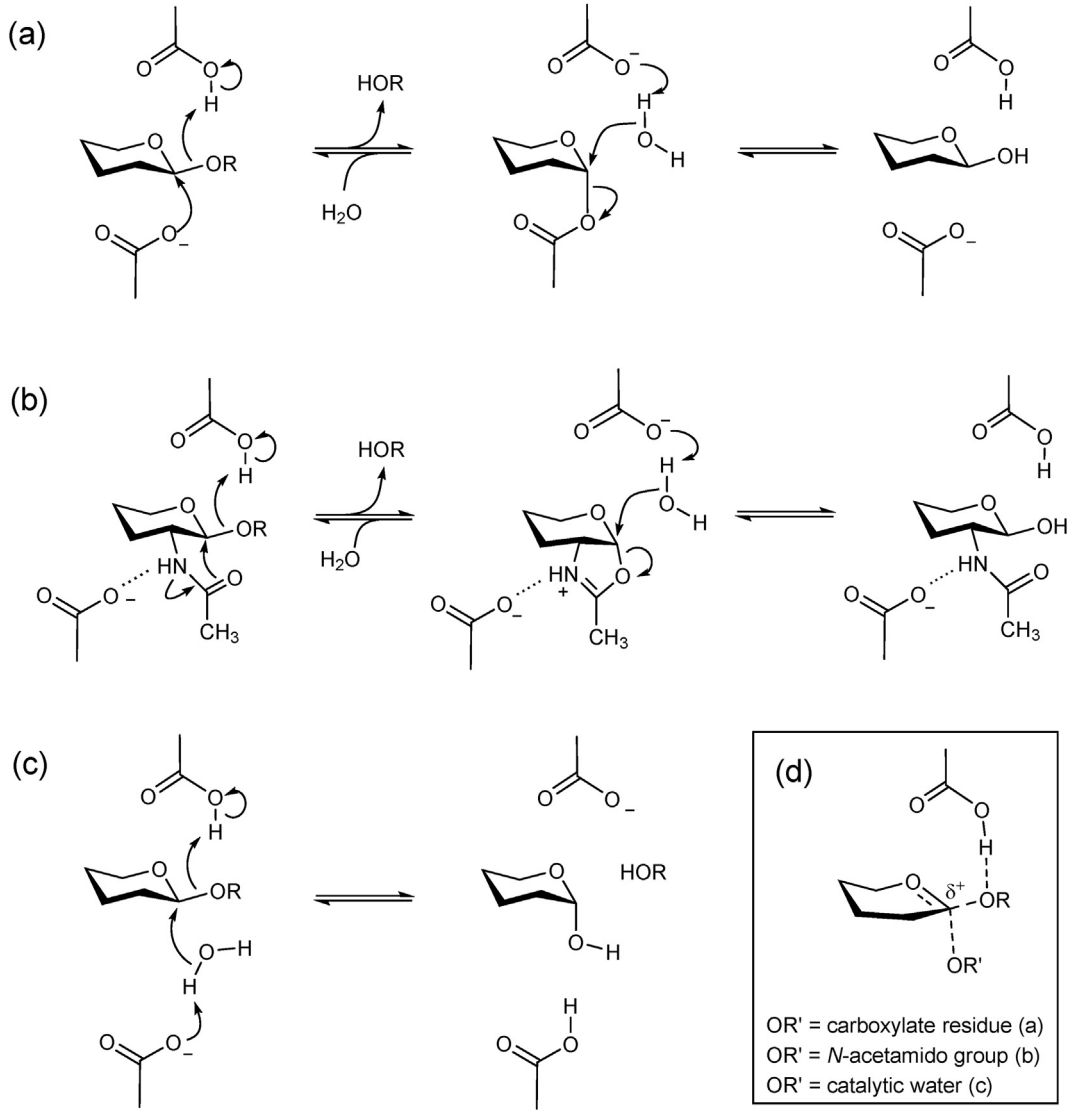
To gain insights into the molecular mechanisms of GH catalysis, it is fundamental to identify the different factors that play a role on these mechanisms, such as specific enzyme–carbohydrate interactions or sugar conformations. A multidisciplinary effort, involving structural and kinetic characterization, together with computational analyses is often necessary [2•]. Computational modeling needs to address enzyme flexibility [3], substrate conformations, and electronic reorganization upon covalent bond cleavage/formation. In the last decade, several complex mechanisms in GHs have been deciphered, in parallel with the enormous increase of computer power and the development of novel modeling approaches. While small models of the active site and static calculations (i.e. optimization of energy along a predefined reaction coordinate) were used in the past, much more realistic models considering the full enzyme and its dynamics are nowadays considered. The *timescale problem*, that is, the fact that chemical reactions take place on a timescale (μs-ms) that is not reachable by room-temperature molecular dynamics (MD) at the quantum mechanics level of theory (ps), can be overcome using enhanced-sampling techniques, such as transition path sampling, umbrella sampling or metadynamics [4]. Here, we highlight some of the work that has been performed in the last few years in the modeling catalytic mechanisms of GHs, using advanced quantum mechanics/molecular mechanics (QM/MM) approaches combined with MD. These are nowadays the methods of choice to capture the essential details of GH catalytic mechanisms and make predictions on yet unresolved mechanistic questions.

Glycoside hydrolases: classical and nonclassical mechanisms

GHs are classified as retaining or inverting enzymes, depending on the stereochemical outcome (Figure 1) [5,6]. The classical (Koshland) retaining mechanism consists in a two-step reaction assisted by two essential

residues: the general acid/base and the nucleophile, which sometimes are difficult to identify [7]. During the first reaction step (glycosylation), the general acid protonates the oxygen of the scissile glycosidic bond, promoting the departure of the leaving group, whereas the nucleophile attacks the anomeric carbon and a covalent glycosyl–enzyme intermediate forms [8]. For some GHs that hydrolyze *N*-acetamido sugars, the carbonyl group oxygen of the *N*-acetyl sugar at the *-1* subsite, rather than the carboxylate group of an enzyme residue, acts as a nucleophile (Figure 1b). This is the so-called substrate-assisted or neighboring group participation mechanism [9], in which an oxazolinium ion/oxazoline intermediate is formed. In the second step (deglycosylation), the acid/base residue acts as a base and deprotonates a water molecule that attacks the anomeric carbon to form the final product. In contrast, inverting enzymes use a single displacement mechanism, in which the general base activates a water molecule, which then performs the nucleophilic attack (Figure 1c). Each reaction step in all these mechanisms is known to involve an oxocarbenium ion–like transition state (Figure 1d), characterized by sp2 hybridization and development of positive charge at the anomeric carbon, as well as significant sugar ring distortion.

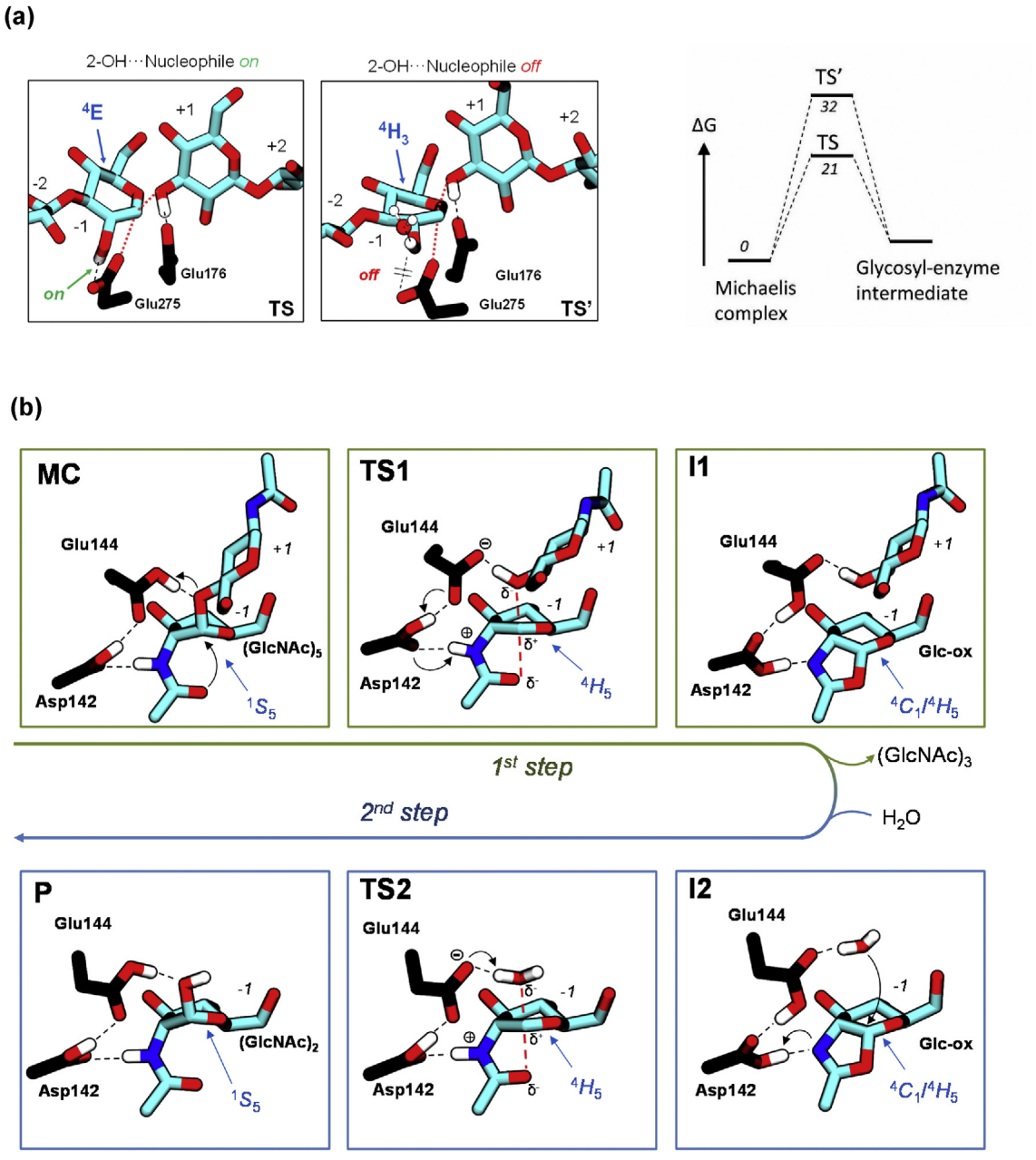
Figure 1



Generalized reaction mechanisms of β-glycosidases. **(a)** Retaining double displacement. **(b)** Retaining substrate-assisted double displacement. **(c)** Inverting single displacement. **(d)** Oxocarbenium ion–like transition state of the first half-reaction. The nucleophilic oxygen that attacks the anomeric carbon is part of a distinct chemical group in (a) to (c). Dashed lines indicate partially broken/formed bonds.

The mechanism of GHs has been the subject of various theoretical investigations. Lysozyme was probably the first GH to be investigated by theoretical methods and was already the focus of the seminal work by Warshel and Levitt [10] in 1976 that set the basis of the QM/MM approach. Lysozyme degrades peptidoglycans by hydrolyzing 1,4-β bonds between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine (GlcNAc). The reaction mechanism, evaluated at that early time with a very simple semiempirical QM method, supported the formation of an oxocarbenium

Figure 2



(a) Changes in transition-state (TS) stabilization of the glycosylation reaction catalyzed by GH72 endo-β-glucanase when the 2-OH···Nucleophile (Glu275) interaction is switched off (a water molecule interacts with the 2-OH in this case) [17]. Only relevant hydrogen atoms are shown. Schematic free energy profile (values in kcal/mol). **(b)** The substrate-assisted reaction mechanism of $(GlcNAc)_5$ hydrolysis by *Sm*ChiB, a chitinase from family GH18, obtained from QM/MM metadynamics simulations [29]. MC = Michaelis complex; I = reaction intermediate state; P = reaction products. I1 and I2 refer to the reaction intermediates, differing on whether the leaving group (*+1* saccharide) or water is present in the active site. The GlcNAc unit at the *-2* subsite is not shown for clarity. Similarly, the GlcNAc units at the *+2* and *+3* subsites is not shown on the top panels (1st reaction step). Only hydrogens attached to heteroatoms are displayed. Bonds being formed/broken are indicated with a dashed red line, whereas relevant hydrogen bonds are indicated by dashed black lines. QM/MM, quantum mechanics/molecular mechanics.

ion intermediate in glycosidic bond cleavage. This proved later to be inoperative in the light of new experiments demonstrating the formation of a glycosyl—enzyme reaction intermediate [8,11], as well as new studies using more advanced QM [12•] and QM/MM methods [13]. The double-displacement reaction has since then been modeled theoretically in a number of GHs using QM/MM approaches (static or dynamic, using either semiempirical or *ab initio* approaches for the QM region) [14]. These studies shed light on mechanistic details that are very difficult to capture by experimental techniques, not only on the nature of the transition state but also on subtle electronic and structural changes along the reaction coordinate, increasing our understanding of the interplay among many factors that contribute to catalysis. For instance, it was shown for GH16 endo-β-glucanase [15], as well as recently for a family GH13 α-glucosidase [16], that the state of maximum anomeric charge occurs after the reaction TS, which is probably not an exception but a general feature. Another study on a retaining endo-β-glucanase from family GH72 [17•] quantified the influence of the hydrogen bond between the 2-OH substituent of the reactive sugar and the catalytic nucleophile (Figure 2a) in the reaction free energy barrier. This effect had been previously estimated by Namchuk and Withers [18] in a GH1 retaining β-glucosidase by kinetic measurements using 2-deoxy substrates. Substituting the 2-OH by 2-H, however, not only affects the 2-OH···Nucleophile interaction. It changes the reaction free energy ($\Delta G^{\ddagger}$) of the glycosylation reaction in two opposite sides: on the one hand, it increases $\Delta G^{\ddagger}$ by suppressing the hydrogen bond interaction (which is more effective at the TS); on the other hand, it stabilizes the charge being developed at the oxocarbenium ion—like TS, decreasing $\Delta G^{\ddagger}$. Raich et al. [17] applied QM/MM metadynamics on GH72 endo-β-glucanase complexed with its natural substrate to determine the net contribution of the 2-OH···Nucleophile hydrogen bond on $\Delta G^{\ddagger}$. By considering two rotamers of the 2-OH (with and without hydrogen bond), it was found that the $\Delta G^{\ddagger}$ of glycosylation and transglycosylation half-reactions increase by 11 and 16 kcal/mol, respectively, when the hydrogen bond is knocked out. These results confirmed the essential role of the 2-OH···Nucleophile interaction in catalysis and further provided insights for modulating an alternative synthetic activity of the enzyme by designing mutations that could affect the interactions of the 2-OH substituent.

Although the classical GH mechanisms (Figure 1) are drawn in terms of a direct attack of the nucleophilic residue on the anomeric carbon, some recent studies reveal that these substitutions can be mediated by water molecules (different from the catalytic water operating in the classical inverting mechanism of Figure 1c). This is not surprising given the known ability of water to translocate protons and rearrange hydrogen bonds via the Grotthuss mechanism. As such, modeling of the reaction coordinate of *Trichoderma reesei* GH6 cellulase [19••], an inverting GH, revealed a chain of two water molecules connecting the reactive saccharide with the general base, which was thereby identified. The simulations were performed using an enhanced-sampling approach (transition path sampling) and a semiempirical method (self-consistent charge density functional tight-binding, SCC-DFTB) to describe the QM atoms. Similarly, simulations on a manno-acting enzyme, *Clostridium perfringens* GH125 exo-1,6-α-mannosidase [20•], using another the metadynamics enhanced-sampling approach and the density functional theory (DFT) method to describe the electronic density of the QM atoms, revealed a similar water-assisted reaction and identified the conformation of the reactive saccharide ring, which was subsequently confirmed by experiments [20•]. Recently, the approach between the acid/base residue and the glycosidic oxygen has been found to be mediated by a water molecule in Autolysin E from *Staphylococcus aureus*, a retaining GH73 *N*-acetylglucosaminidase [21]. The QM/MM MD simulations were performed using a replica path method to activate the chemical reaction and DFT to describe the QM atoms. Interestingly, an exhaustive analysis of the dynamics of the acid/base residue in the Michaelis complex captured two distinct scenarios on its interaction with the glycosidic oxygen. It interacts either directly or indirectly (via a water molecule), but the conformation with indirect (i.e. water-assisted) interaction is the most reactive one. Clearly, slight mechanistic variations from the classic Koshland ones are emerging as more thoughtful QM/MM MD analyses are being performed.

Other GHs that exhibit mechanisms far beyond the classical Koshland mechanisms have been reviewed by Withers [22]. For instance, family GH4 α- and β-glucosidases follow redox and elimination steps involving NAD^{+} and a divalent metal as cofactors, and its detailed mechanism remains unclear [23]. Family GH33 sialidases use an elimination and hydration mechanism involving an activated tyrosine residue as a nucleophile [24—26]. GH99 endo-α-mannosidases have been shown to follow a two-step mechanism in which an assisting Glu residue acts as a general base to deprotonate the 2-OH, facilitating a nucleophilic substitution at C1 and leaving group departure [27]. This novel mechanism of glycosidic bond cleavage has been recently demonstrated by a combination of theory and experiment [28••].

In spite of the recent advances in the understanding of complex GH mechanisms, there is still debate even regarding aspects of well-characterized mechanisms. A QM/MM metadynamics investigation on GH18 chitinases, GHs that follow a *substrate-assisted* mechanism (Figure 1b) [29•], identified a hydrogen bond network operating at the active site (Figure 2b) that favors a neutral oxazoline, rather than an oxazolinium ion as previously assumed [30,31], as a reaction intermediate.

This scenario cannot be extended to all GHs that operate via similar substrate-assisted catalysis. A diversity of protonation states of the reaction intermediate is likely to be expected, depending on the local environment of the basic residue that interacts with the oxazoline nitrogen atom (Figure 1b), influencing its pKa.

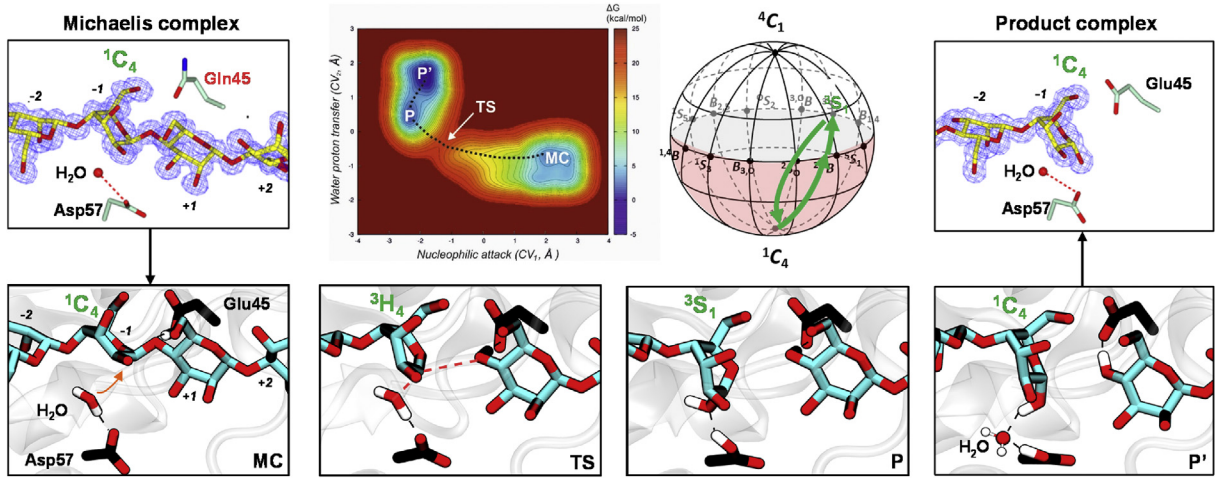
Cellulases, found in many GH families, have long since attracted the attention of computational biochemists for its applications to biomass degradation and the biofuel industry. Reilly et al. [32•] investigated the mechanism of family GH8 endoglucanase using QM/MM metadynamics based on DFT. The simulations reproduced the concerted one-step general inversion mechanism, confirming the identity of the general base residue and the boat-type conformation of transition state. Retaining cellulose hydrolysis has been investigated in a family GH7 cellobiohydrolase [33•]. Using transition path sampling and the SCC-DFTB method, the authors modeled the complete reaction pathway for cellulose hydrolysis (both glycosylation and deglycosylation) and could correlate it with the structural information at different stages of the reaction. It was found that deglycosylation proceeds via a product-assisted mechanism wherein the glycosylation product, cellobiose, positions a water molecule for nucleophilic attack on the anomeric carbon of the glycosyl–enzyme intermediate, in contrast with the common view that deglycosylation occurs once the product is out of the catalytic pocket.

The last few years have witnessed the discovery and characterization of enzymes that greatly speed up the breakdown of cellulose and biomass in general [34]. Lytic polysaccharide mono-oxygenases are copper-dependent enzymes able to hydrolyze recalcitrant polysaccharides using oxygen and reducing agents. Their molecular mechanisms are being elucidated at atomic level using QM/MM techniques, showing the presence of highly reactive radical species that ultimately lead to the hydroxylation of the polysaccharide anomeric carbon [35,36,37•].

Predicting conformational catalytic itineraries

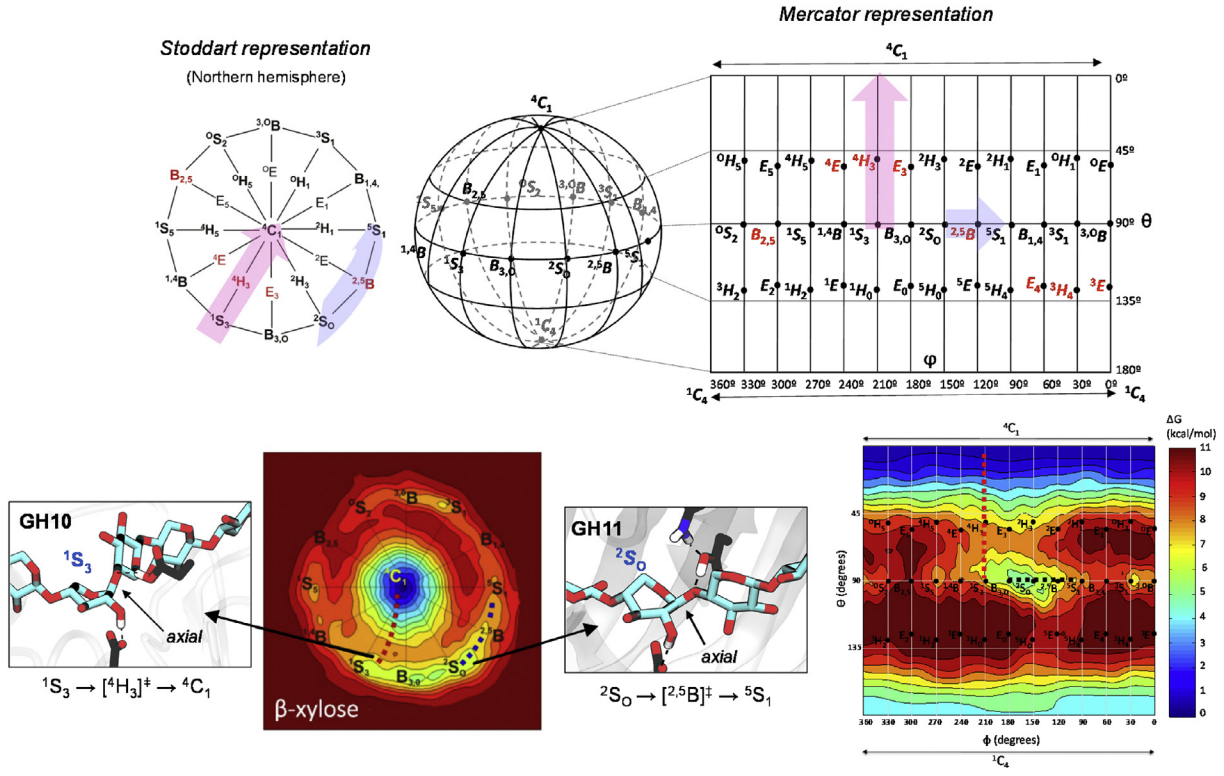
One important aspect of GH mechanisms is the conformation of the saccharide ring at the *-1* subsite (the '*-1* sugar') during the catalytic reaction, that is, the *conformational catalytic itinerary*. In the case of retaining GHs, the *itinerary* normally refers to the glycosylation half-reaction, which is typically the rate-determining step, especially for sugar-like substrates. The catalytic itinerary, particularly the *-1* sugar conformation at the TS, is important for the design of inhibitors and activity-based probes [38,39] (also refer to the study by Overkleeft and Davies in this issue). Even though the conformational itinerary might not be a perfect longitudinal (or latitudinal) pathway across the Cremer-Pople sphere [6] (the conformation of the *-1* saccharide during glycosylation in a family 16 β-glucanase was early found to be warped line around the 'canonical' $^{1}S_{3}$

Figure 3



Catalytic mechanism of *Ss*GH134-inverting endo-β-1,4-mannanase. The crystal structure of the Michaelis complex (PDB 5JUG) was connected end-to-end with the one of the products (PDB 5JU9) by means of QM/MM metadynamics, obtaining the free energy landscape of the reaction and unveiling a southern hemisphere itinerary [2]. The catalytic acid Glu45 has been mutated to Gln45 in the Michaelis complex crystal structure to prevent hydrolysis. This mutation was reverted in the simulations, and the conformation of the substrate was monitored along the reaction. From P to P′, a solvent water molecule enters the active site at the same time that the saccharide at the *-1* subsite changes conformation from $^{3}S_{1}$ to $^{1}C_{4}$, matching the conformation observed in the crystal structure. Hydrogen atoms have been omitted for clarity, except those of the catalytic water and the carboxylate group of Glu45. Three collective variables were used to describe the bonds that break and form during the reaction, including the proton transfer between Glu45 and the glycosidic oxygen, the nucleophilic attack of the water molecule, and the proton transfer with Asp57. QM/MM, quantum mechanics/molecular mechanics.

Figure 4



(Top) Stoddard and Mercator representations of the puckering sphere (top-center picture) of a pyranose ring. Conformations highlighted in red are favored conformations for an oxocarbenium ion, and thus possible transition states of glycosylation reactions. The two main itineraries that have been experimentally proposed for β-xylanases are shaded in pink (itinerary proposed for family GH10 β-xylanases) and violet (itinerary proposed for family GH11 β-xylanases). (Bottom) Conformational free energy landscape of an isolated β-xylose in both Stoddard (left) and Mercator (right) representations. Independent of the type of representation used, the low free energy regions of the FEL delineate the two favored conformational catalytic itineraries for β-xylanases: $^1S_3 \rightarrow [^4H_3]^‡ \rightarrow {}^4C_1$ and $^2S_O \rightarrow [^{2,5}B]^‡ \rightarrow {}^5S_1$. FEL, free energy landscape.

$\rightarrow [^4H_3]^‡ \rightarrow {}^4C_1$ itinerary expected for retaining β-glucanases [15]), computational studies show that it is not very different from it [14]. Determination of conformational itineraries is also useful to understand the strategies that nature has evolved to overcome the stereochemical challenges associated with glycan hydrolysis and transfer. This is particularly relevant for GHs acting on α- or β-mannosides, in which the 2-hydroxyl group occupies an axial orientation in the undistorted $^4C_1$ chair conformation. α-Mannosides do not need sugar distortion to facilitate catalysis, as the leaving group adopts an axial orientation, being preactivated for nucleophilic attack from the β-face. However, the axial 2-OH makes the approach of the nucleophile (either a protein residue in retaining enzymes or a water molecule in inverting enzymes) from the β-face difficult. Thus, α-mannosidases commonly distort the substrate in a conformation in which the 2-OH is equatorial and the leaving group is axial. In the case of β-mannosidases, this avoids the cis-1,2-diaxial clash. These GHs have been shown to use $B_{2,5}$ or $^3H_4$ conformations in their transition states. The corresponding itineraries have been successfully characterized by QM/MM simulations, such as work on family GH38 Golgi α-mannosidase II ($B_{2,5}$ transition state) [40] and family GH47 α-mannosidase I ($^3H_4$ transition state) [41]. Recently, a GH134-inverting β-mannosidase was found to feature an anomalous inverted-chair conformation ($^1C_4$) at the Michaelis and product complexes. This seemed incompatible with the expected $^1S_5 \rightarrow [B_{2,5}]^‡ \rightarrow {}^OS_2$ itinerary of β-mannosidases and suggested a new itinerary in the "South-Pole" of the Cremer-Pople puckering sphere, $^1C_4 \rightarrow [^3H_4]^‡ \rightarrow {}^3S_1$. But the observed product complex conformation ($^1C_4$) did not match this itinerary either. This conundrum was solved by QM/MM metadynamics simulations using three collective variables to describe the three sets of covalent bond pairs being broken/formed. The computed reaction coordinate (Figure 3) was consistent with a $^1C_4 \rightarrow [^3H_4]^‡ \rightarrow {}^3S_1$ itinerary, novel for β-mannosidases, and captured the relaxation of the −1 sugar conformation from $^3S_1$ to $^1C_4$ immediately after the reaction, thus connecting the snapshots that had been obtained by X-ray analyses (Figure 3) [2••]. This study illustrates distinct solutions

to the problem of nucleophilic attack on a β-manno-pyranoside residue.

Conformational catalytic itineraries can not only be obtained from modeling of the reaction coordinate, as shown previously, but also be predicted from much simpler calculations on isolated sugars. It was excitingly discovered one decade ago that the conformational free energy landscape (FEL) of a single pyranose (e.g. β-glucose or β-mannose) encodes information of the most likely conformation of that sugar when it is on-enzyme, as well as the catalytic itineraries for GHs acting on substrates that are derivatives of that given sugar (e.g. β-glucosidases or β-mannosidases, respectively). Biarnés et al. [15] found that the conformations observed in Michaelis complexes of β-glucosidases lie in low-energy regions of the FEL of isolated β-glucopyranose and can be predicted by analyzing the electronic and structural properties of these conformers. Similar conclusions were reached later in a study based on static DFT calculations on a number of ring conformations (i.e. energy calculations followed by geometry optimization and free energy corrections) [42]. Metadynamics simulations of sugar puckering have often been applied to several monosaccharides with significant predictive outcome [14]. An interesting example is β-xylose (Figure 4), whose FEL nicely reflects the two itineraries proposed for β-xylosidases ($^{1}S_{3} \rightarrow [^{4}H_{3}]^{\ddagger} \rightarrow {}^{4}C_{1}$ and $^{2}S_{O} \rightarrow [^{2,5}B]^{\ddagger} \rightarrow {}^{5}S_{1}$) and was used to exclude another previously proposed pathway [43]. From a methodological point of view, these FELs are obtained by using pyranose ring puckering coordinates [44] as collective variables in a metadynamics algorithm, which can be done either in the form of Stoddart diagram (i.e. Cartesian coordinates, projecting either the North or the South hemisphere) or in a Mercator representation (Figure 4). The possibility of obtaining the complete FEL in a single simulation and its easiness of interpretation makes it a very practical strategy for predicting/discarding GH conformational itineraries. Recently, these analyses are being applied to understand GH inhibition. Specifically, the FEL of a given sugar-like inhibitor informs on the preferred conformation and the accessibility of secondary conformers that can lower inhibition efficiency [39,45].

Future prospects

Computer simulations have been fundamental in the understanding of the chemistry underlying GH mechanisms, identifying the catalytic residues, validating controversial conformational itineraries and capturing important mechanistic details that escape experimental probes. Still, the catalytic mechanism of several GH families remains unexplored. A challenge in the field is to describe the enzyme active site reorganization during substrate binding, that is, how does the substrate acquire its functional distorted conformation upon binding to the enzyme and how does the enzyme change in this process. GHs are known to be generally robust, but exo-acting enzymes or GHs active on two different type of substrates (e.g. Os7BGlu26 β-glycosidase is active on both gluco- and manno-configured substrates [46]) have very plastic active sites. Elucidating the interplay between protein and substrate conformational changes is relevant for the function of glycosyltransferases, CAZymes responsible for glycosidic bond synthesis, with mechanisms that are related to those of GHs [14,47]. These enzymes have quite open active sites, in which substrate binding often depends on flexible lectin domains [48]. Recent work on the autocatalytic reaction of the glucose-storage enzyme glycogenin exemplifies how an active site/enzyme dynamically adapts to multiple size substrates to facilitate the chemical reaction [49•]. We can anticipate that, in the next years, MD and hybrid QM/MM MD simulations will continue deciphering complex and fascinating CAZyme mechanisms, providing hints for enzyme engineering and inhibitor design.

Conflict of interest statement

Nothing declared.

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