

Understanding functional dynamics and conformational stability of betaglycosidases

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CHAPTER 1

Retaining β -glycosidases GH5, GH30 and GH11: Folds, Dynamics, Stability and Inhibition

Abstract

etaining β-glycosidases are a ubiquitous family of enzymes found in all kingdoms of life. They are produced by living organisms to catalyze the hvdrolvsis of carbohvdrates β -glycosidic links in a highly sophisticated manner, thus they are involved in a plethora of biological processes. In fungi and bacteria, they are used to process organic biomass as source of energy and also to metabolize carbohydrate conjugates for structural and host infection purposes. In mankind, their functions or dysfunctions are correlated with multiple metabolic disorders, such as lysosomal storage disorders and lactose intolerance. Among the largest families of retaining β -glycosidases are GH5, GH30 and GH11. These hydrolase families include most of the known β-glycosidases in nature with different substrate specificities and structural topologies. GH5 and GH30 belong to Clan A hydrolases with a $(\beta/\alpha)_8$ TIM barrel catalytic domain, while GH11 belongs to Clan C with a β -jelly roll fold. All of the three families have found multiple biotechnological applications and the quest for discovering new inhibitors against them has been an intense research area. In this introduction, the aim is to discuss the general characteristics of GH5, GH30 and GH11 fold topologies and classification, in addition to the dynamics and stability of their catalytic domains. Besides, it is aimed to introduce the most important inhibitors and discuss their applications in fundamental research, biotechnology and therapeutic fields.

Introduction

arbohydrates in their diverse forms in conjunction to their glycoconjugates are cornerstone components of life in all organisms. Besides their structural and energy storage roles, they are involved in a myriad of biological processes, including -but not limited to- cell communication, host-pathogen interactions¹, signal transduction, inflammation², intracellular trafficking and multiple diseases³. In nature, plant cell walls represents the largest reservoir of carbohydrates, forming about 75% of the biomass on earth⁴. The functional and structural diversity of carbohydrates is a consequence of the astronomic number of possible combinations of their monosaccharides building blocks⁵, therefore it is not surprising that the genomes of most organism include about 1-3% genes that code for carbohydrate-active enzymes⁶. Polysaccharides are very stable compounds in nature; their spontaneous hydrolysis occurs at a rate in the range of 10⁻¹⁵ s⁻¹, corresponding to a half-life of 4.7 million years⁷! Organisms have evolved to produce highly efficient catalysts that hydrolyze carbohydrate glycosidic bonds and enhance the hydrolysis rate by a factor of more than 10^{17} ⁷. This superfamily of sophisticated enzymes is called glycoside hydrolases (GHs), and its members exert their function in a specific manner on various glycosidic bonds depending on the number, position, or configuration of the hydroxyl groups in the sugar molecule. Therefore, glycoside hydrolases have found multiple biotechnology applications, such as in biofuel production^{θ}, paper pulp bleaching and the food industry^{θ}. In humankind, deficiency of GHs leads to multiple metabolic disorders such lysosomal storage disorders¹⁰ and lactose intolerance¹¹. Hence, glycosidases inhibition has been an attractive subject of research, and the identified inhibitors have found extensive use as agrochemicals and therapeutic agents¹², such as antifungal agents, insecticides, antidiabetics, anti-obesity compounds, antivirals and compounds to correct lysosomal storage disorders^{13, 14}. To discover novel glycosidases from genomic sequences, an enormous effort was dedicated to classify this historically important family of enzymes using several classification systems, including those based on the substrate or product specificity, mode of attack (exoglycosidic versus endo-glycosidic enzymes) and the stereochemical mechanism. In 1991, a classification of glycoside hydrolases based upon amino acid sequence similarities was introduced, which is now provides more than 140 sequence-based families of glycoside hydrolases, and structures of members of at least 120 families have been solved $(www.cazy.org)^{15}$. Among the largest GH families are the retaining β -glycosidases GH5, GH30 and GH11. Although these three families differ in terms of structures and sequences, all of them include members with the same substrate specificity, such as β glucosidases, endo- β -1-4 xylanases and β -glycosylceramidases from different organisms¹⁶. 17

B-glycosidases have variable structures and substrate specificities, therefore, each GH family has been classified into different Clans¹⁸. The GH5 and GH30 β -glycosidases classify as the GH Clan A, which consists of proteins with an (β/α)₈-barrel catalytic domain fold, while GH11 is in the GH Clan C, with a β -jelly roll catalytic domain fold. Due to their industrial and therapeutic importance, the focus here is to discuss the general features of their fold topology, dynamics, conformational stability and inhibition, with the aim to provide the reader with a general overview of the structural and biophysical characteristics of these three families of enzymes.

β-glycosidases catalytic mechanism

Retaining β -glycosidases perform their catalytic reaction in two steps first described by Koshland, using a catalytic dyad functioning as a nucleophile and acid/base¹⁹. The first step is a glycosylation of the enzyme by nucleophilic attack of the anomeric carbon of the substrate, which induces an electronic rearrangement and a hydrolysis of the glycosidic linkage. The nucleophilic attack is concomitant with a proton transfer from the acid/base residue and the departure of the leaving group from the aglycon site. In the second step, an activated water molecule acts as a nucleophile with the assistance of the general acid/base to deglycosylate the nucleophile residue. The product is released from the glycon pocket and a new catalytic cycle could take place (Figure 1.1.a). It is proposed that the two steps of the reaction pass through a transient oxocarbenium like state and depending on the sugar ring configuration the substrate could adopt different itineraries pathway during catalysis¹⁹. In the case of GH5 and GH30, the substrate itinerary is supposed to pass by the ${}^{3}S_{1} \rightarrow {}^{3}H_{4} \rightarrow {}^{1}C_{4}$ pathway for the Michaelis complex \rightarrow TS \rightarrow intermediate enzymatic half reaction²⁰ (Figure 1.1.b). On the other hand, the substrate itinerary for GH11 xylanases has been proposed to follow a path of ${}^{4}C_{1} \rightarrow {}^{0}E \rightarrow {}^{0}S_{2}{}^{\sharp} \rightarrow$ $B_{2,5}$ for the xylose residue at -1 subsite²¹. However, this issue remains as yet unresolved. Early structures of intermediate complexes trapped with 2-fluoro sugars were interpreted as ${}^{2,5}B$ conformations²², suggesting a ${}^{2}S_{O} \rightarrow {}^{2,5}B \rightarrow {}^{5}S_{I}$ itinerary. The Michaelis complex of xylohexaose bound to the xylanase XynII from T. reesi revealed a slightly distorted ${}^{4}C_{1}$ conformation²¹. Thus, the sugar ring itinerary of GH11 families number is still under debate.

Retaining β -glycosidases can also perform a transglycosylation reaction by simply changing the temperature²³ or the pH²⁴ of the enzymatic assay or by providing the enzyme with a suitable aglycon acceptor²⁵ (**Figure 1.1a**). This reaction has been successfully applied to synthesize oligosaccharides and glycoconjugates. The transglycosylation capacity of (mutant) β -glycosidases, in combination with their high regio- and stereospecificity, makes them an attractive instrument to synthesize complex carbohydrates.



Figure 1.1. (a) General hydrolysis and transglycosylation mechanism and (b) Koshland double displacement mechanism and substrate itinerary of retaining β -glucosidases.

GH5 and GH30 fold topology

All GH5 and GH30 glycosidases have a $(\alpha/\beta)_8$ -TIM barrel catalytic domain with two conserved carboxylic acid residues located in β -strands 4 and 7, serving as nucleophile and acid/base catalytic dyad. The distance between these two catalytic residues is highly conserved, often between 5 and 5.5 Å between the O_{ϵ_1} and O_{ϵ_2} atoms of the nucleophile and acid/base glutamic acid residues, respectively²⁶. GH5 and GH30 glycosidases comprise fungal, bacterial and eukarvotic β -1-4 glucanases, β -1-4 mannases, β -1-4 xylanases, cellulases and glucosylceramidases^{16,17}. GH5 glycosidases have been further classified into 53 subfamilies providing a more accurate prediction of function of yet uncharacterized proteins¹⁷. A new classification approach has led to the transfer of five GH5 protein subgroups to GH30 group 2, including the lysosomal enzyme GBA (Figure **1.2c**). The GH5 and GH30 glycosidases resemble each other regarding protein structure and substrate specificities, but show differences in topology^{16,27}. For instance, one characteristic of the GH30 enzymes is the fusion of the $(\alpha/\beta)_{8}$ -barrel catalytic domain with a β -structure consisting of an immunoglobulin like fold (**Figure 1.2f**). This β structure, poorly conserved in GH30 glycosidases, is absent in GH5 enzymes (Figure 1.2d). Characteristically, the TIM barrel of most GH5 enzymes is sealed with a cap like structure, which does not occur in GH30 enzymes (Figure 1.2d). A special case is endoglycoceramidase II (EGCII) from Rhodococcus sp. that removes the entire oligosaccharide from gangliosides like GM3 and GM1 (Figure 1.2d)²⁸. EGCII has twofold domains, a catalytic TIM barrel adjoined to a β -sandwich domain, like in GH30 members. However, the β -structure domain differs from the ones observed in most GH30

enzymes, being composed of only 8 β-strands in a barrel geometry (**Figure 1.2e**). Exceptionally, the TIM barrel of EGCII is not capped by the small β-strand sheet observed in most other GH5 family members²⁹. Unlike EGCII, EGCI (another endoglycoceramidase from *Rhodococcus equi* with broader substrate specificity than EGCII) displays a typical GH5 TIM barrel fold sealed with two β-strands at the non-catalytic face of the domain. However, in addition, the TIM barrel of this enzyme is also fused to a β-sandwich structure³⁰ (**Figure 1.2a**). Thus, EGCI is a typical GH5 enzyme while EGCII shows structural features of members of both GH5 and GH30 families. The evolutionary relationship between these enzymes remains unclear.

In humans, no endoglycoceramidase is characterized yet, although an endo hydrolysis activity towards gangliosides has been noted for human cancer cells and tissues of other mammals^{31,32}.



Figure 1.2. GH5 and GH30 general fold and secondary structure arrangement. (**a**) EGCI 3D structure (PDB ID: 5j7z)³⁰. (**b**) EGCII 3D structure (PDB ID: 2osx)²⁹. (**c**) GBA 3D structure (PDB ID:2v3e)³³. (**d**) Diagram of the GH5 family members secondary structure organization adopted from (16). (**e**) EGCII secondary structure arrangement (PDB: 2osx). (**f**) GBA (PDB: 1ogs) secondary structure elements of GH30 members¹⁶. β # refers to TIM barrel domain β -strand number and β s# refers to a β -strand number of the β -side domain.

GH11 fold topology

GH11 is one of the best characterized GH families, which is reflected by the 31 structures deposited in the PDB and CAZyme databases. This family includes bacterial and fungal enzymes considered to be true xylanases, due to their high substrate specificity toward non-decorated xylan polymers and their high catalytic efficiency³⁴. GH11 enzymes display unique biophysical properties such as a low molecular weight (20 kDa) and variable pH and temperature optima, making them attractive candidates for several industrial and biotechnological applications³⁵. For instance, xylanases are widely used in paper pulp bleaching³⁶ biofuel production^{θ}, and food industries⁹. This class of enzymes has a globular catalytic domain with an approximate gyration radius of 17 Å and a β jelly-roll fold composed of antiparallel β -sheets A and B that shape a long (~25 Å), deep (~9 Å) and wide (4 Å) catalytic cleft³⁷ (Figure 1.3a). The overall shape of this fold is often compared to a right-hand fist³⁷. The β -strands A are nearly planar and distorted at the end to form a hand palm and fingers, and the B strands are in a perpendicular position to the A strands sculpting the binding cleft including, the thumb region. The two sides of the cleft are connected by a long loop called the $cord^{3\beta}$. The fold includes a single α -helix, sitting under the β -strands B structure. Around 20 % of the amino acids comprizes serines and threenines, providing an extensive hydrogen bond network that rigidifies the fold. A sequence alignment of the GH11 family members, using 236 unique sequences out of 456 PSI-BLAST hits (the 220 excluded sequences where found to be more than 95%) conserved) revealed a high conservation, (Figure 1.3b) most variability is observed for the surrounding residues located on the protein surface. The residues lining the catalytic cleft are highly conserved, both in space and in sequence, and a "backbone" like pattern is formed by multiple aromatic side chains at the bottom of the cleft (Figure 1.3a). Among the aromatic residues (residue numbering refers to 1BCX), many are highly (more than 80%) conserved regarding the type of the aromatic side chain (Trp9, Tyr69, Trp71 and Tyr80) while other positions seem to be more flexible (Trp129 and Tyr174 can be substituted by Phe and Trp, respectively). Tyr165 is more variable (less than 50% conserved) and can sometimes be substituted by Glu, which could mean that a polar group is more important than an aromatic residue at this position.

The PSIXG sequence motif of the thumb region is highly conserved, suggesting the importance of this hairpin loop for the protein function (**Figure 1.3a**). The amino acid sequence that harbours the nucleophilic glutamic residue is also conserved (ATEG) with one exception, where a glutamine is present instead of a glutamic acid. The sequence in which the acid/base residue (Glu) is located is more variable, and in some cases, the acid/base residue is substituted by either a glutamine or an arginine. These type changes of the catalytic dyad could reflect a different mode of action of these enzymes or a change of their pH optima. Overall, the strong sequence conservation of the GH11 family is remarkable because generally, proteins structures are better conserved than sequences³⁹.

GH11 family members exhibit a wide variability of their biochemical properties with different pH and temperature optima. These variabilities seem to be caused by only minor sequence differences.



Figure 1.3. (a) GH11 β -jelly-roll fold. (b) GH11 amino acid conservation based on sequence alignment using the ConSurf Server.

β-glycosidases dynamics and stability GH5 and GH30 TIM barrel dynamics and stability

Enzymes are very dynamic entities, exerting their function by sampling multiple conformational substates during catalysis⁴⁰. Thus, it is insufficient to describe only the ground state structure of the resting state if a complete understanding of the catalytic function is to be achieved. Although there are numerous β -glycosidases structures available, few efforts were dedicated to investigating their molecular dynamics. The scarcity in dynamics studies could stem from the robust understanding of the chemistry that governs β -glycosidases catalytic reactions and also from the challenges of studying protein dynamics experimentally. The biophysical and theoretical techniques that have been employed to study the global dynamics of retaining β -glycosidases include molecular dynamic simulations (MD), frequency decay fluorometry that exploit tryptophan emission, hydrogen/deuterium exchange (HDX) mass spectrometry and the versatile technique NMR spectroscopy, which can cover a broad spectrum of protein time scale motions from ps to hours with an atomic resolution.

Clan A β -glycosidases have a TIM barrel catalytic domain fold, so studying the dynamics of a particular member could provide a general trend for the dynamics of this recurrent topology. For instance, David *et al.* have studied the dynamics of the catalytic domain of the β -(1,4)-glycosidase Cex (or CfXyn10A) from the soil bacterium *Cellulomonas fimi* from ns to ms time scale using NMR spectroscopy⁴¹. The conclusion from this study was that the TIM barrel domain is uniformly rigid on the different probed time scales. Few dynamic changes were observed for the glycosyl-enzyme intermediate state complex,

although an enhancement of the thermal stability and proteolysis resistance of the domain was observed. A lower resolution technique was also employed to follow the conformational dynamics of a thermophilic and a mesophilic Clan A β -glycosidase form extremophilic archaeon Sulfolobus solfataricus (Sßgly) and from Escherichia coli (Cßgal), using multitryptophan emission decay⁴². The thermophilic variant harbours twelve tryptophans distributed over the core of the protein TIM barrel domain and some of them are located on flexible loops. The study showed that there are two groups of tryptophan fluorescence decay: A short-lived and a long-lived tryptophan life time decay, reflecting the well packed and rigid TIM barrel core and the flexibility of the protein surface loops, respectively. However, when incubated with the irreversible inhibitor cyclophellitol (see below), a quenching of tryptophan short lived residues fluorescence was observed, pointing toward a structural rigidification of the TIM barrel flexible loops. In contrast, the mesophilic β galactosidase variant exhibits a uniform distribution of tryptophan fluorescence decay, an indication of a looser structure of the TIM barrel domain. The same trend was also observed in case of β-glucosylceramidase (GBA) where HDX mass spectrometry was employed. The study showed that the residues that reside within the core of TIM barrel domain are highly protected against the H/D exchange process, and loops that connect the α -helices and β -sheet of the catalytic domain are the ones with the lowest protection factor⁴³. All of these observations lead to the conclusion that the TIM barrel domain is globally rigid, yet includes flexible loops that might have a determinant role in protein function. It is proposed that the rigid TIM barrel fold provides the correct frame for favourable interactions with carbohydrate substrates and this rigidity could be necessary to bind, distort, and subsequently hydrolyze the glycosidic linkage within the enzyme active site⁴¹.

GH11 β -jelly roll dynamics and stability

The β -jelly roll fold is common among a variety of proteins with no functional correlation³¹. To interrogate GH11 β -jelly roll fold dynamics and the contribution of the protein motion in its catalytic reaction β -1-4xylanase form *Bacillus circulans* (BCX) was selected to be studied by NMR due to its well-characterized structure and suitable biophysical properties⁴⁴. BCX comprizes 185 residues with a molecular weight of 20 kDa and a stokes radius of ~17 Å³⁷. Its small size and well-dispersed NMR spectra facilitated a thorough study of its dynamics. A survey carried out by Connelly *et al.* has revealed that the protein in its substrate-free form displays a high average order relaxation parameter (S² =0.86+/-0.04), indicative of restricted motion on the pico-nanosecond time scale, which is in agreement with the very compact and globular fold observed with crystallographic and hydrodynamic experiments⁴⁵. The dynamics uniformity of BCX is in line with the extensive intramolecular hydrogen bond network. By examining the BCX crystal structure, 146 backbone amides out of 185 participate in this network. The same

dynamic behaviour on the fast timescale was observed when studying the complex of the protein with 2-deoxy-2-fluoro- β -xylobioside. However, these findings do not exclude the possibility of slower dynamics on the millisecond time scale, which is more relevant for catalysis as it covers the rate of the substrate turnover (20 s⁻¹ against 2,5-DNPX2 substrate)⁴⁶.

A unique feature of GH11 is the presence of a thumb loop region that is believed to play a determinant role in selective substrate binding and product release47. Multiple molecular dynamics simulations have suggested the opening of the thumb loop to induce the product dissociation⁴⁸⁻⁵⁰. The thumb region displays a well-structured classical hairpin, containing a type I β -turn (residues 117-120 in BCX) with six internal hydrogen bonds. The only evidence of a full opening of the thumb was observed in the case of a specific xylanase mutant from *B. subtilis* in the crystalline state⁵¹. A recently published study of xylanase millisecond time scale dynamics, using the relaxation dispersion CPMG technique⁵², showed that the protein in its free form experiences a millisecond motion for multiple residues localized within the binding site⁵³. Residues of the thumb regions were not detected due to peak broadening. In the presence of its substrate, an enhancement of the millisecond (ms) time scale motion was observed. This study has provided the first evidence of such motion in GH11 family enzyme. It is difficult to interpret relaxation dispersion effects in structural terms. These ms dynamics might reflect a chemical exchange of the backbone amides that could be due to side chain reorientations and not due to conformational changes in the protein backbone.

A thorough MD investigation on GH11 family enzymes was undertaken for XYNII from *Trichoderma reesei*⁵⁴. By combining the different crystal structures, it was proposed that the protein samples three sequential substates during its catalytic reaction. First, the protein adopts an open state to allow substrate accommodation within the cleft. Substrate binding induces a closed conformation necessary for positioning the substrate into the correct configuration for glycosidic bond hydrolysis. The last state is a loose structure from which the product is released. The study provided a model for the enzyme catalytic cycle based on the available crystal structures that still needs to be validated with studies in solution.

Overall, the conclusion that could be deduced from these studies is that the β -jelly roll catalytic domain of GH11 has an inherent structural rigidity emerging from the strong hydrogen network between its secondary structure elements. Most of the GH11 family members are secreted enzymes⁵⁵. Thus, the lack of their conformational flexibility might be an adaption mechanism to function in harsh extracellular conditions. Besides, no allosteric regulation was identified for GH11 xylanases so far. Therefore, it is speculated that they do not need to contain flexible regions to facilitate effector binding. Additionally, this structural rigidity could also be required for sugar ring distortion necessary for the hydrolysis of the glycosylic link. To this point, all of the experimental observations point toward a globular and rigid fold of GH11 during its reaction pathway with minor

conformational changes of the protein structure and a plausible local opening of the thumb region.

β-glycosidases inhibitors and their applications

The high abundance of β -glycosidases encoding genes in all organisms is reflecting their pivotal roles in multiple biological processes. In humankind, β -glycosidases function or dysfunction is related to different pathological states, leading to an increased interest in the therapeutic application of inhibitors. Cognate β -glycosides inhibitors can be divided in different classes based on their mode of action and covalency. In the following section, the focus is on the most frequently used β -glycosidases inhibitors.

<u>Covalent mechanism-based inhibitors</u>: Mechanism-based inhibitors are covalent inhibitors (or inactivators) that obliterate the enzyme activity by forming a covalent bond between the enzyme and a functional group of the inactivator⁵⁶. The bond is typically formed by a nucleophilic attack of an activated center on the inactivator leading to the formation of a covalent complex. Inactivation can be caused by blocking the substrate accessibility to the enzyme active site or by modifying one of the enzyme catalytic residues. This class of inhibitors has attracted interest to probe β -glycosidases structure and function and to identify catalytic residues⁵⁷.

Epoxide- and aziridine-based inactivators. This type of inactivators has found successful applications due to their high inactivation efficiency and stability. Aziridine and epoxide are widely utilized to identify glycosidases nucleophile catalytic residues as they exploit the first step of the catalytic enzyme mechanism. The inactivation mechanism involves the attack of the epoxide or aziridine by the enzyme nucleophile, resulting in ring opening and the formation of an adduct bond between the enzyme and the inhibitor, which emulates the intermediate state of the natural substrate hydrolysis reaction. The process is facilitated by protonation of the inactivator by the general acid/base residue (Figure 1.4a)⁵⁷. In 1974 Quaroni et al. have used conduritol B-epoxide (CBE) 1 (Figure 1.4a) to identify the catalytic carboxylate in both sucrase and isomaltase⁵⁸. Radioactive CBE derivatives were also used to identify catalytic active site residues in different glycosidases⁵⁹ before bioinformatics emerged as a predictive method. The structural symmetry of CBE allows it to label both α and β -glycosidases^{60,61}. Moreover, CBE has been shown to interact not only with nucleophile residues involved in catalysis but in some cases also with other active site residues. For instance, identification of the β -lac Z, β glucosylceramidase and almond β-glucosidase nucleophile residues by CBE was initially mistaken due to the inhibitor promiscuity, and it was later corrected^{62,63}. Epoxide derivatives, such as exo-alkyl epoxide glycosides 2 (Figure 1.4a), a type of molecule that contains an epoxide moiety linked to a glucoside by an alkyl chain, were also effectively

used to label the nucleophile catalytic residue for a variety of enzymes and X-ray crystallography was used to solve the 3D structure of their complexes⁶⁴. It was observed that the linked residue depends on the alkyl chain length, such as in retaining β -1-4-xylanase from *T. reesi*, in which 2,3-epoxypropyl β -D-xyloside and 3,4-epoxybutyl β -D-xyloside labelled the nucleophile and the acid/base residues, respectively⁶⁵. It was speculated that these results are mainly due to the alkyl chain flexibility and the different inactivator orientations adopted within the enzyme active site. The aza-analogue of CBE (aziridine) **3** (**Figure 1.4a**) was found to be a moderate inactivator for both Abg β -glycosidase and yeast α -glucosidase, with slightly higher activity against the latter⁶⁶. Aziridine based inactivators exhibit a positive charge, thus, they are specifically directed toward the negative active site of the enzyme, precluding non-specific interactions with other parts of the protein. These compounds have been shown to be more reactive than their epoxide counterpart due to the lower stability of the aziridine cycle⁶⁶.

CBE was extensively used to study and characterize human β -glucosylceramidase (GBA) structure/function relationships^{68,69}. Aerts and co-workers have used CBE in the discovery of a novel cytosolic human GBA⁷⁰. Irreversible binding of CBE to GBA was exploited to knock out GBA activity in both cell cultures and animals, allowing the study of GBA roles in maintaining cellular homeostasis and to establish the role of glucosylceramides accumulation in cancer cell^{71,72}. CBE was also used to produce cellular and animal models for Gaucher disease and to study the morphology of GBE into healthy mice has helped to evaluate the minimum GCase activity (12-16%) required for maintaining a correct cellular functioning⁷⁵.

Although CBE has proven to be an effective β -glycosidases inactivator, its inherent symmetry limits its specificity toward different β -glycosidases. Thus, it has been proposed that the addition of a hydroxymethylene group on the C5 of CBE pyranose ring would enhance its potency and selectivity toward β -glycosidases⁶⁵. Indeed, a natural compound identical to the proposed CBE derivative has been extracted from the mushroom Phellinus sp. by Atsumi *et al.*⁷⁶, and it showed to be selective exclusively toward β -glycosidases. The compound was called cyclophellitol 4 (Figure 1.4a) and it has been used in the same manner as CBE against human GCase to induce a Gaucher disease-like state in cell cultures and in animal model^{77,78}. The crystal structure of cyclophellitol covalently bound to a β -glycosidase has given clear insight into the intermediate state sugar ring distortion of this family of enzymes. The compound has been shown to adopt a ${}^{4}C_{l}$ chair conformation⁷⁹. These new structural insights have guided Witte et al. to rationally design selective activity-based probes (ABPs) toward GBA by incorporating a reporter group (biotin or BODIPY) attached via an alkyl or acyl linker to the cyclophellitol scaffold or its aziridine analogue^{g_0}. The ABPs have found various biological applications and further β and α -glycosidases ABPs have been synthesized based on this principle^{81,82}.



Figure 1.4. Retaining β -glycosidases mechanism-based inhibitors. (a) Epoxide- and aziridine-based inactivators. (b) CBE inactivation mechanism.

Activated fluorinated glycosides inhibitors: The activated 2-deoxy-2-fluoro glycosides 5 (**Figure 1.4a**) are one of the most selective retaining β -glycosidases inactivators. These inactivators mimic the natural substrate of the enzyme by incorporating an activated aglycon leaving group linked to a glycoside with a fluoride at the position C2. Thus, they are usually considered as slow substrates. The occupancy of C2 position by a fluoride helps to stabilize the intermediate state by destabilizing the oxocarbenium ion like transition states (**Figure 1.5**) $^{\beta\beta}$. The spontaneous hydrolysis of the enzyme-glycosyl intermediate has variable rates and its lifetime can vary from seconds to months. The enzyme catalytic activity could be rescued by using a suitable acceptor to transfer the fluorinated glycoside and liberate the nucleophile residue⁸³. Like CBE and cyclophellitol, activated fluorinated glycosides have been extensively used to investigate the structure/activity relationship of a variety of retaining β -glycosidase enzymes using various biophysical techniques⁸⁴. Additionally, NMR spectroscopy has been employed to generate a model of the oxocarbenium ion-like transition state itinerary and to investigate the dynamic aspects of β -glycosidases during the catalytic pathway, using this class of inactivators as a tool^{41,85}.

Fluorinated glycosides have also found multiple biological applications. They were used to identify novel mammalian retaining β -glucosidases⁸⁶ and to characterize the mammalian lactase phlorizin hydrolase binding pocket⁸⁷. The ability to trap β -glycosidases in their intermediate state has also been used to explore the role of their aglycon binding site and its specificity. The kinetics of recovery of the enzyme activity,

after complex formation with fluorinated glycosides, was shown to be related to the binding affinity of the acceptor to the aglycon site^{*89*}. This class of "slow substrates" was also used as a warhead to generate ABPs by linking the compounds to a fluorogenic reporter^{*89*}. These ABPs were successfully used to selectively label β -glycosidases in complex biological samples and to discover novel β -glycosidases enzymes in *Cellulomonas fimi*⁹⁰.



Figure 1.5. The inactivation mechanism of activated fluorinated glycoside inhibitors for retaining β -glycosidases.

<u>Non-covalent inhibitors</u>: Non-covalent β -glycosidases inhibitors are more therapeutically attractive as they can reversibly inhibit the enzyme activity in a dose-dependent manner. Therefore, several β -glycosidases inhibitors comprising disaccharides, iminosugars, cabasugars, thiosugars and non-glycosidic inhibitors were either synthesized or extracted from natural sources⁹¹. In the following section, the focus is mainly on the iminosugars inhibitors because of their successful therapeutic application in a variety of human lysosomal storage disorders.

Iminosugars are low molecular weight compounds also called polyhydroxylated alkaloids that exhibit at least two hydroxyl groups and one heterocyclic nitrogen atom. These compounds are abundant in plants and microorganisms⁹². The substitution of the oxygen or anomeric carbon of the pyranose ring by a protonated nitrogen has been proposed to mimic the positive charge generated in these positions upon partial cleavage of the glycosidic bond, thus emulating the transition state, which triggers a high inhibition potency⁹³ (**Figure 1.6b**).

One of the first iminosugars described was nojirimycin (NJ) **6** (Figure 1.6a), isolated from several strains of *Bacillus* and *Streptomyces* as well as mulberry tree leave. It contains an endocycle nitrogen in the place of the oxygen pyranosidic atom, and it was shown to be a potent inhibitor of both α and β -glycosidases inhibitors⁹¹ However, the presence of C1 hydroxylic group renders this compound unstable, hindering utilisation. 1-Deoxynojirimycin (DNJ) **7** (Figure 1.6a) is a reduced version of nojirimycin that lacks the hydroxyl group from the anomic carbon enhancing the stability and inhibition

potency of the compound⁹¹. This compound also acts on both β and α -glycosidases. Changing the position of the heterocycle nitrogen atom to the anomeric carbon position of the DNJ pyranosidic ring led to a 440-fold inhibition enhancement toward β -glycosidases with a moderate inhibition toward α -glycosidases⁹⁴. It has been proposed that this enhancement of the inhibition potency is due to the better ability of the new compound in mimicking the β -glycosidases transition state. The new analogues are named 1-azasugars, represented by isofagomine (IFG) **8** (**Figure 1.6a**). IFG has the anomeric carbon and the ring oxygen of glucose replaced by nitrogen and carbon, respectively, and the C2 hydroxylic group is absent. The remaining hydroxyl groups are maintained, preserving a D-glucose like configuration. However, it was suggested that by keeping the hydroxyl group at C2 a stronger β -glycosidases inhibition would be observed, presumably due the addition of a hydrogen bond interaction with the enzyme active site. The compound having these characteristics is named noeuromycin **9**, and it showed high inhibition specificity toward β -glycosidases⁹⁵.

The superior specificity of azasugars toward β -glycosidases has prompted their use as potential therapeutic substances, especially in the case of the lysosomal storage disorder such as Gaucher disease⁹⁶. GBA is the enzyme responsible for cleaving the glucosidic bonds between the glucose and ceramide moieties of glucosylceramides in the lysosomal compartment of the cell. Reported single point mutations in GBA have been shown to decrease the half-life of the protein by destabilizing its native fold or by leading to the production of a misfolded protein. The deleterious GBA mutants are degraded by the cell control machinery, thus hampering the enzyme from reaching its lysosomal destination and leading to the accumulation of its glucosylceramide substrate%. IFG is one of the best structurally characterized azasugars, and due its high specificity and potency toward GBA, it was proposed to be used as a pharmacological chaperone to rescue GBA proteostasis in GD patients. In vitro and in vivo studies provided evidence for its stabilization effects on multiple GBA variants⁹⁷. For instance, IFG was found to have a higher binding affinity to GBA at neutral pH (as found in the ER compartment) compared to acid pH (as found in the lysosomal compartment)⁴³. This pH binding dependency is a pivotal property in assisting the folding of GBA mutants in the ER compartment to escape the cellular quality control machinery and to be released in the lysosome. IFG increases the thermal stability of the enzyme at neutral pH and raises the cellular level of GBA N370S and L444P mutants in GD patients tissues⁹⁸. IFG is supposed to cross the blood-brain barrier and correct GD manifestation in the nervous system due its small size. Despite these promising results, IFG has failed in clinical trials, presumably due to its hydrophilicity, resulting in poor biodistribution. Thus, N-alkylated derivatives were produced and demonstrated to have a superior chaperone performance⁹⁸. One of the successful compounds is N-butyl-deoxynojirimycin (NB-DNJ, Miglustat) 10 (Figure 1.6b), which has dual beneficial effects on glucosylceramide depletion and on the

stabilization of GBA mutants⁹⁹. Another derivative is N-nonyl-deoxynojirimycin (NN-DNJ) **11** (**Figure 1.6b**), which showed an enhanced binding affinity toward GCase when tested *in vivo*, presumably due to its longer alkyl chain length¹⁰⁰. Tuning of the alkyl chain length of these compounds and elucidating the stabilization mechanism toward GBA might provide valuable information that could pave the way for the development of a new generation of pharmacological chaperones.



Figure 1.6. Retaining β -glycosidases iminosugar inhibitors. (**a**) Different type of iminosugars used against retaining β -glycosidases as reversible inhibitors. (**a**) Isofagomine inhibition mechanism.

Conclusion and perspective

The structure/function relationship studies of β -glycosidases have provided an enormous amount of information about their physical characteristics. Uncovering the substrate itinerary during catalysis has helped to produce more potent and efficient inhibitors, although the full potential of this information has yet to be explored for a large number of retaining β -glycosidases, and, besides, the inhibitory stabilization mechanism is still poorly understood.

Despite these tremendous insights, understanding of retaining β -glycosidases dynamics in relation to substrate binding and distortion as well as product release is still limited. Studies of the dynamics could address the paradox between the high structural conservation and wide variety of functions. The unique characteristic of β -glycosidases to shift the equilibrium of their hydrolysis reaction toward polymer synthesis under specific conditions has encouraged their use to produce relevant carbohydrates with a wide spectrum of therapeutic, industrial and research applications. Enzymatic synthesis of carbohydrates offers a one-step reaction under mild conditions with stereospecificity and without by-product generation and less environmental pollution, compared to classical chemical methods, which still require several protection, activation, coupling and deprotection steps.

Depletion of fossil fuels at an enhanced rate and the effect of its utilization on the global economy and environment have pressed the quest for a renewable and clean source of energy. Bioethanol has been considered as a potential alternative fuel as its raw material (cellulose) is highly abundant¹⁰¹. β -glycosidases hold a promising future regarding their use in cellulose bioconversion process and biofuel production¹⁰². Fundamental studies of this family of enzymes will help to compose the right cocktail of enzymes for maximum cellulose bioconversion and to guide their re-engineering for optimal function under large scale industrial conditions.

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THESIS OUTLINE

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The work presented in this thesis is devoted to deepening our understanding of the functional dynamics and conformational stability of retaining β -glycosidases. Over the years, tremendous efforts were dedicated to elucidate the structure/function relationship of this ubiquitous family of enzymes and to discern their substrate itineraries during catalysis. Nevertheless, the mechanism of stabilization of β -glycosidases by active site binders and the contribution of dynamics in catalysis remains poorly explored. Therefore, in this thesis it is aimed to address these aspects, relying on activity-based probes technology in conjunction with standard biochemistry and advanced NMR techniques. **CHAPTER 1** gives a general introduction of the biophysical properties of GH5, GH11 and GH30 β -glycosidase families and the inhibitors used to modulate their functions, both in fundamental research and in therapy. CHAPTER 2 describes the application of ABPs to characterize the conformational stability of endo-glycoceramidase EGCII, a bacterial homologue of GBA that holds promise as a therapeutic application against a number of inherited glycosphingolipidoses caused by deficiency in lysosomal exoglycosidases. **CHAPTER 3** describes the application of ABPs and reversible β -glycosidase inhibitors to dissect the stabilization mechanism of GBA by active site occupancy in vitro and in vivo, as a proof of the principal for the pharmacological chaperone treatment against Gaucher disease. **CHAPTER 4** describes the conformational landscape and dynamics of β glycosidases and their possible roles in the catalytic mechanism, using GH11 xylanase as a model. **CHAPTER 5** gives general conclusions of this work and future perspectives.