Glycoside Hydrolases: Catalytic Base/Nucleophile Diversity

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ABSTRACT: Recent studies have shown that a number of glycoside hydrolase families do not follow the classical catalytic mechanisms, as they lack a typical catalytic base/ nucleophile. A variety of mechanisms are used to replace this function, including substrate-assisted catalysis, a network of several residues, and the use of non-carboxylate residues or exogenous nucleophiles. Removal of the catalytic base/ nucleophile by mutation can have a profound impact on substrate specificity, producing enzymes with completely new functions.

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Introduction

Glycoside hydrolases (GHs), a widely distributed group of enzymes, cleave glycosidic bonds in glycosides, glycans and glycoconjugates, and they can play key roles in the development of biofuels and in disease research. GHs such as cellulases, xylanases, and other glucosidases are being used to produce sugars from pretreated biomass substrates, which are then fermented to produce ethanol or butanol as renewable alternatives to gasoline (Wilson, 2009). Glycosidases also participate in a broad range of biological processes including the virulence of pneumococci, which cause a number of serious diseases that are responsible for millions of deaths annually (Abbott et al., 2009). Therefore, glycosidase inhibitors have great therapeutic potential, including providing a treatment for Alzheimer's disease (Yuzwa et al., 2008). These enzymes also function in the carbon cycle to allow microorganisms in the soil to break-down plant cells, releasing CO_2 aerobically and various fermentation products anaerobically (Bardgett et al., 2008).

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Knowledge of the catalytic mechanisms of GHs will help to design highly effective inhibitors and produce more active enzymes. Based on sequence similarities and predicted structures, GHs are classified into 113 families in the database: Carbohydrate Active enZYmes, or CaZy (www.ca-zy.org) (Cantarel et al., 2009). The database is updated frequently; for instance, GH-115 has been recently added (Ryabova et al., 2009) while five other families GH-21, 40, 41, 60, and 69 (Cottrell et al., 2005; Smith et al., 2005) were deleted due to their lack of glycosidic bond hydrolysis. This classification system allows prediction of the catalytic mechanism and key catalytic residues as these are conserved in most of the GH families (Henrissat et al., 1995).

GH families are categorized into two classes: retaining or inverting (Koshland, 1953), depending on the change in the anomeric oxygen configuration during the reaction. A typical inverting glycosidase requires a catalytic acid residue and a catalytic base residue while a typical retaining glyco-sidase contains a general acid/base residue and a nucleophile (discussed below). Identification of catalytic residues is essential to understand the catalytic mechanism of glyco-sidases. This review focuses on the identification of "enzy-matic nucleophiles," which are defined as electron pair donors, that is, the catalytic base residue in inverting GHs as well as the nucleophile in retaining GHs. The diversity of catalytic mechanisms that have been identified shows that the classical view of Koshland is not always correct, which suggests the need to investigate novel mechanisms and opens the possibility of engineering enzymes for new functions.

Proposed GH Catalytic Mechanisms

In inverting GHs, the catalytic acid residue donates a proton to the anomeric carbon while the catalytic base residue removes a proton from a water molecule, increasing its nucleophilicity, facilitating its attack on the anomeric center. In retaining GHs, a general acid/base catalyst works first as

an acid and then as a base in two steps: glycosylation and deglycosylation, respectively. In the first step, it facilitates departure of the leaving group by donating a proton to the glycosyl oxygen atom while the nucleophile forms an enzyme sequestered covalent intermediate. In the second step, the deprotonated acid/base acts as a general base to activate a water molecule that carries out a nucleophilic attack on the glycosyl–enzyme intermediate, with the two inversion steps leading to retention of the stereochemistry at the anomeric center (Fig. 1).

One of these catalytic mechanisms is conserved among all members of each GH family, except for GH-23 (Davies and Sinnott, 2008), GH-83 (Morley et al., 2009), and GH-97 (Kitamura et al., 2008), which have both inverting and retaining members, as well as GH-31, which contains typical retaining α-glucosidases and α-1,4-glucan lyases that use a β-elimination mechanism (Lee et al., 2003). In some extreme cases, a GH might have properties of both catalytic mechanisms; for instance, a *Thermobifida fusca* inverting GH-43 has recently been shown to have trans-glycosylation activity (József Kukolya, personal communication). A GH-4 6-phospho-α-glucosidase from *Bacillus subtilis* acts by a retaining mechanism except on substrates with activated leaving groups, where it acts as an inverting glycosidase (Yip et al., 2007).

A number of related families show conservation of the detailed catalytic mechanism and structure, forming GH-clans, named by letters from A to N (Davies and Sinnott, 2008). This grouping takes advantage of the available information on the catalytic mechanisms of member enzymes.

Ways to Identify the Enzymatic Nucleophile

Structure-Related Approaches

The availability of three-dimensional structures of representatives from 85 families helps to identify enzymatic nucleophiles as these residues are located in the active site cleft, and are generally conserved, polar and hydrogen-bonded (Bartlett et al., 2002). The detection of the nucleophilic water molecule in the atomic-resolution crystal structure of unliganded enzymes supports the identification of the catalytic base residue, particularly when that residue has an H-bond to the nucleophilic water. Structures of
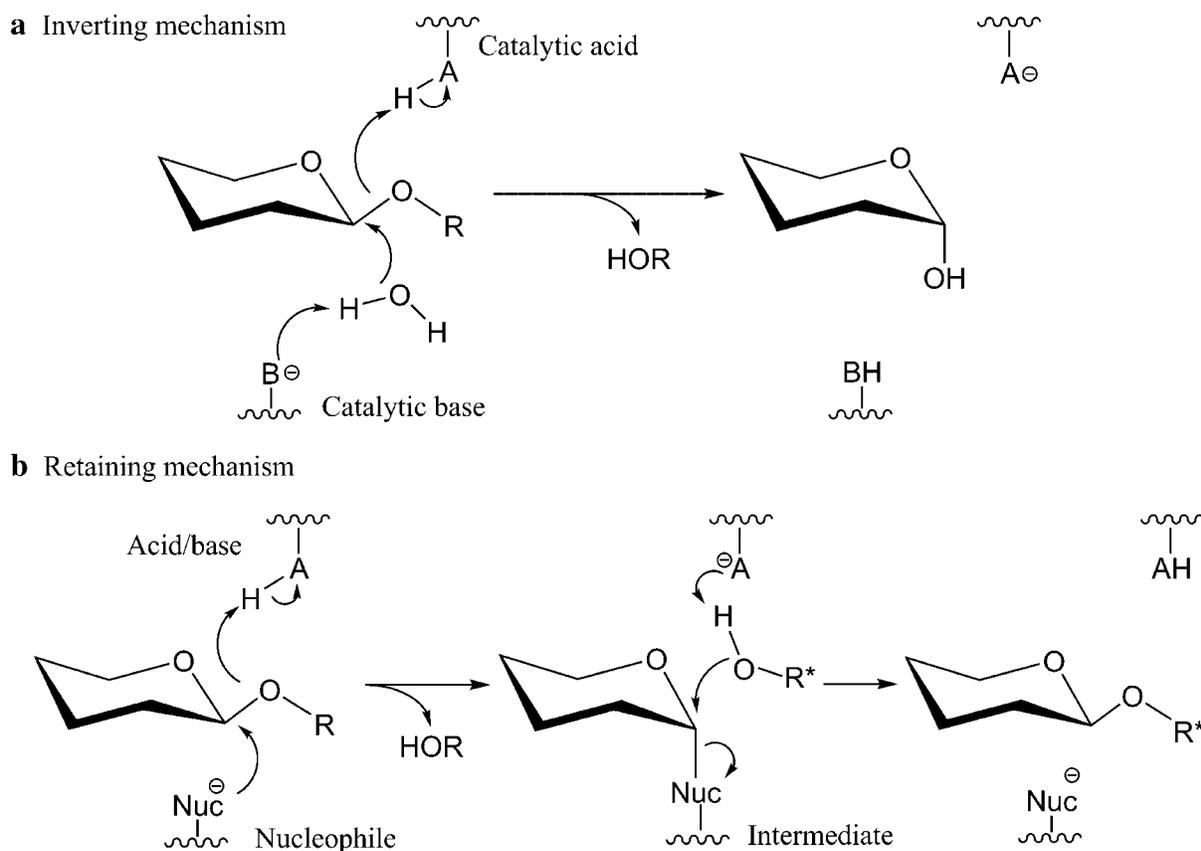

Figure 1. Proposed inverting (**a**) and retaining (**b**) mechanism. AH: a catalytic acid residue, B-: a catalytic base residue, Nuc: a nucleophile, and R: a carbohydrate derivative. HOR*: an exogenous nucleophile, often a water molecule.

unliganded glycosidases can be used for ligand docking and computational simulations to identify catalytic residues. Docking several short ligands into the active site of an inverting GH-43 xylosidase (Jordan et al., 2007) and an inverting GH-47 α-mannosidase (Karaveg et al., 2005) identified candidate catalytic bases. Catalytic residues stand out in a computational titration simulation as they often more readily donate or receive protons, and therefore ionize at a different pH than most comparable residues (Sterner et al., 2007). Evidence for a catalytic base residue was also obtained by first-principles quantum mechanics/molecular mechanics simulations (Petersen et al., 2009), which allowed observation of proton transfer from the nucleophilic water to the putative base residue in the transition state.

Many glycosidases have been crystallized with ligands or substrates, facilitating identification of the nucleophilic residue, which is located near the anomeric center. In retaining GHs, glycosidic hydrolysis is conducted via two separate steps with the formation of a glycosyl–enzyme intermediate, which can be trapped using fluorinated substrate analogs (Guce et al., 2010) to identify the nucleophile (Fig. 2). Additionally, the intermediate can be trapped by flash freezing (Lunin et al., 2004) or by mutating the acid/base residue (Damager et al., 2008). A combination of mass spectrometry and mechanism-based fluorescent labeling reagents such as 4-N-dansyl-2-difluoromethylphenyl β-galactoside, which inactivate both retaining and inverting GHs, can capture and identify catalytic residues (Kurogochi et al., 2004).

Biochemical Approaches

Availability of 3D structures, development of algorithms with a high accuracy of up to 90% in the prediction of catalytic residues (Tang et al., 2008) as well as the conservation of catalytic mechanisms in GH families greatly help predicting a catalytic base/nucleophile. However, a similar fold does not necessarily imply a similar function (Sadreyev et al., 2009) or a similar catalytic mechanism (Kitamura et al., 2008). It is difficult to formulate the relationship between enzyme structure and bond specificity as minor changes alter this. Additionally, flexibility of loops containing catalytic residues also can cause difficulty for structure-based prediction (André et al., 2003). Therefore, biochemical assays are crucial to confirm the identification.

Mechanistic studies using kinetic isotope effects (KIEs), which provide probes of transition state structure (Lee et al., 2003; Yip et al., 2007), coupled with trapping a covalent glycosyl–enzyme intermediate are an effective approach to identity the nucleophile (see Vocadlo and Davies (2008) for a review of KIEs).

Site-directed mutagenesis of conserved carboxylic acids followed by kinetic analysis with substrates bearing different leaving groups, pH dependence profile and chemical rescue experiments are commonly used to identify catalytic residues. In a catalytic base mutant of an inverting GH, enzymatic activity is very low and it is not affected by substrates with different leaving groups. In contrast, removal of the acid residue inactivates hydrolysis of substrates with poor leaving groups but not those with good leaving groups. This approach has been used to propose the catalytic base in an inverting GH-6 cellulase from *Cellulomonas fimi* (Damude et al., 1995), and to identify the acid residue in an inverting GH-6 exocellulase from *T. fusca* (Vuong and Wilson, 2009). However, the method is not an absolute test for a base residue in inverting GHs, as any residue that is important for substrate binding or pK_a modulation of either the catalytic acid or base residues, would show the same pattern of activity as a real base. Supporting evidence for the presence of a catalytic base residue in inverting GHs can be from replacement of candidate residues with cysteinesulfinate followed by oxidation to cysteinesulfinic acid (Cockburn et al., 2010), but this is not a definitive approach. In retaining GHs, the aglycon group is cleaved during the first step of the reaction; therefore, the rate of glycosylation is
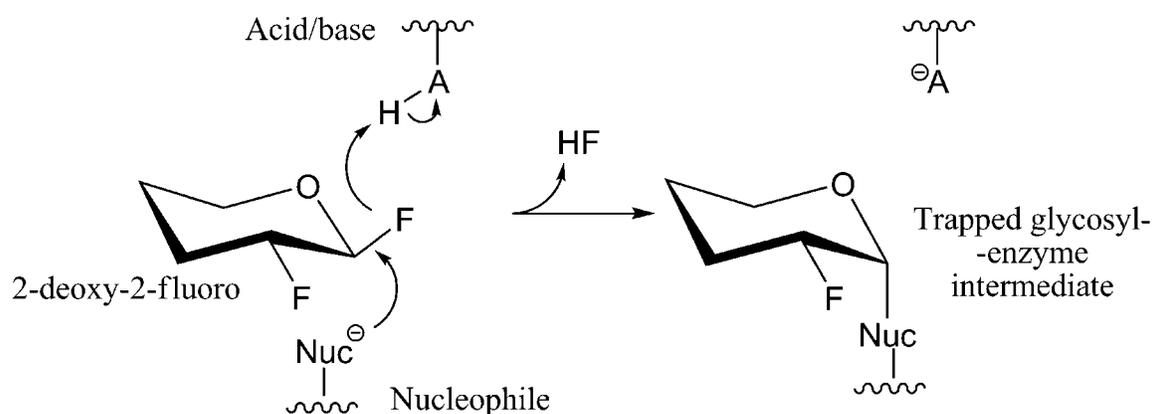

Figure 2. Trapping a glycosyl–enzyme intermediate using 2-deoxy-2-fluoro sugars.

influenced by the pK_a of substrates. The use of substrates with different leaving groups is often applied together with Brønsted plots, which allows the quantification of the negative charge developed on the glycosidic oxygen in the transition state of inverting enzymes, indicating the difference in proton donation ability between a catalytic acid mutant and wild-type enzyme (Shallom et al., 2005).

The pH profiles of glycosidases are typically bell-shaped, mainly reflecting the ionization state of the two carboxylic catalytic residues in the active site (Collins et al., 2005). Replacement of a nucleophile or other catalytic components usually alters the corresponding ionization in the pH profile.

The addition of exogenous nucleophilic anionic compounds, typically sodium azide or sodium formate to mutant enzymes can provide unambiguous evidence for identification of both catalytic residues in retaining GHs (Comfort et al., 2007) and the catalytic base in inverting GHs (McGrath et al., 2009). These exogenous anions can act directly on the glycosidic bond to form an adduct or indirectly through a water molecule (Nagae et al., 2007) (Fig. 3). In retaining GHs, sodium azide could rescue the activity of enzymes with mutations in either the acid/base residue or the nucleophilic residue, yielding a glycosyl-azide product with different anomeric configuration (Comfort et al., 2007). Chemical rescue can be ion specific; therefore, rescue experiments should be conducted with several exogenous nucleophiles. Steric hindrance in the active site, the tight spatial requirements of an external nucleophile or dramatic change in the pH profile due to a catalytic mutation make chemical rescue experiments not always effective.

Identification of catalytic bases/nucleophiles is not always successful. A number of single mutations in proposed catalytic base residues of inverting GHs resulted in reduced or undetectable activity while crystallographic analysis suggested that these residues could not directly access the nucleophilic water (Nagae et al., 2007) or they are involved in catalysis only after a conformational rearrangement (Davies et al., 2000). Specific mechanism-based labeling combined with mass spectrometry identified two residues that were labeled with a suicide-type fluorescent substrate in a *Vibrio cholerae* neuraminidase; however, these residues are approximately 20 Å away from the known catalytic pocket (Hinou et al., 2005). Studies in *T. fusca* endocellulase Cel6A (André et al., 2003) and human cytosolic sialidase Neu2 (Chavas et al., 2005) showed that their key residues for catalysis moved significantly upon substrate/inhibitor binding (Fig. 4).

In contrast, structural analyses and pH-activity profiles clearly identified a residue as a catalytic base, but its mutation did not cause a drastic loss of activity (Collins et al., 2005). A phylogenetic study showed that the location of the catalytic base in inverting GH-8 enzymes can vary (Adachi
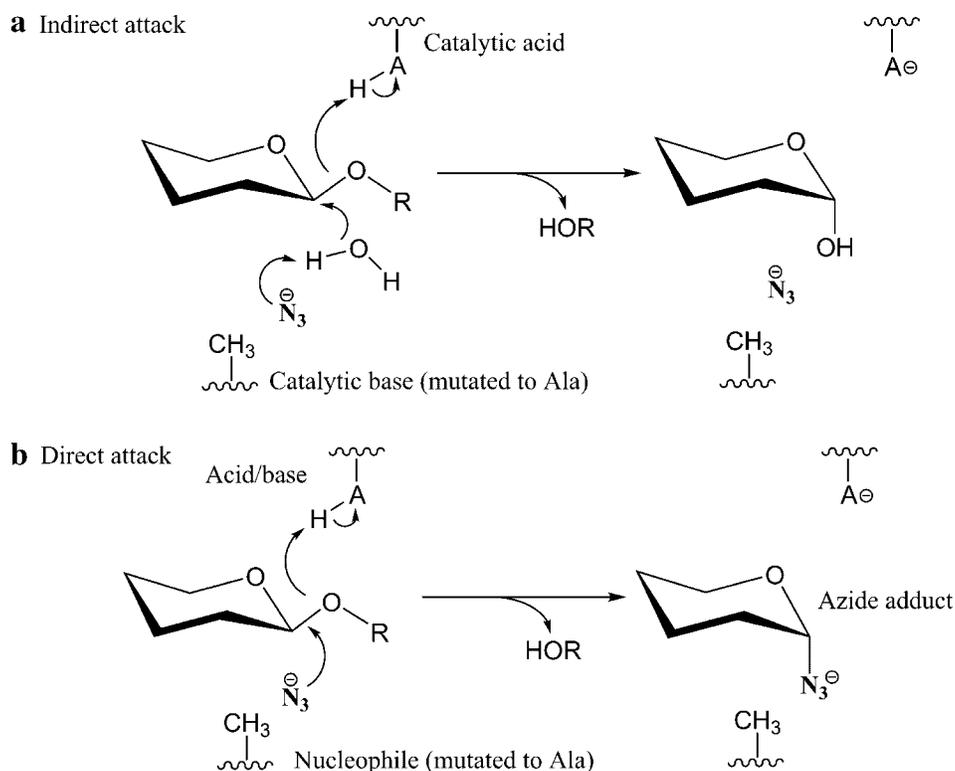

Figure 3. Indirect and direct attack of sodium azide on the anomeric center for activity rescue.
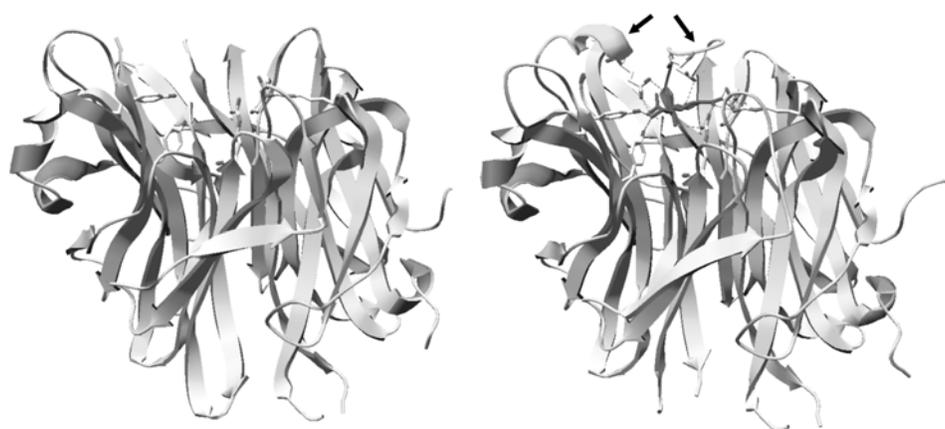
Figure 4. Conformational changes of human cytosolic sialidase Neu2 after inhibitor binding. Left: Ribbon diagram of Neu2 apo form. Right: Two new helices (arrows) and H-bonds (dots) formed after DANA binding.

et al., 2004). In a *Clostridium thermocellum* GH-8 endo-cellulase CelA, Asp278 was suggested by structural analyses as the base catalyst (Guérin et al., 2002); however, site-directed mutagenesis of this residue did not reduce cellulase activity (Yao et al., 2007). A single residue may possess more than one role in catalysis, particularly for enzymes using elimination steps. A Tyr in a retaining GH-4 β-glycosidase was proposed to play the double role of general base in C2 deprotonation as well as general acid/base in assisting C1–O1 bond cleavage/formation (Varrot et al., 2005). Another factor is that the catalytic residues can be located in different domains as in retaining GH-3 β-D-glucan glucohydrolase (Hrmova and Fincher, 2007) or even in different monomers of a homotrimeric enzyme as in a *Shigella flexneri* phage Sf6 endorhamnosidase, which is a retaining glycosidase, but has not been classified into any GH family yet (Müller et al., 2008). Despite these difficulties, the number of inverting GHs with a known catalytic base and retaining GHs with a known nucleophile are increasing, that is, an inverting GH-9 *T. fusca* processive endocellulase (Li et al., 2007), an inverting GH-81 *T. fusca* laminarinase (McGrath et al., 2009) and a retaining GH-93 *Fusarium graminearum* exo-1,5-α-L-arabinanase (Carapito et al., 2009).

What Replaces a Typical Catalytic Base/Nucleophile?

A number of GH families do not have a typical carboxylate base/nucleophile, but use novel mechanisms to replace them (Table I), demonstrating the complexity of glycoside-cleaving mechanisms.

Substrate-Assisted Mechanisms

The presence of a nucleophile in retaining GHs is important as it directly attacks the anomeric center to form a glycosyl–enzyme intermediate. However, by both detailed kinetic and X-ray structural studies, the carbonyl oxygen of the 2-acetamide group in the substrate of GH-18 chitinases (Honda et al., 2004), GH-20 hexosaminidases (Langley et al., 2008), GH-56 hyaluronidases (Markovic-Housley et al., 2000), GH-84 *O*-GlcNAc-ases (Dennis et al., 2006), GH-85 *endo*-β-*N*-acetylglucosaminidases (Ling et al., 2009; Rich and Withers, 2009), and GH-103 lytic transglycosylases (Reid et al., 2007) has been shown to act as the nucleophile to form an oxazoline intermediate (Fig. 5). Recently, this mechanism

Table I. Substitutes for a single carboxylate base/nucleophile in GHs.

|	Substitute	Representative GHs	Refs.
Substrate-assisted catalysis	2-Acetamide, polysialic acid	GH-18, 20, 56, 58, 83 (inverting), 84, 85, 103	Dennis et al. (2006); Honda et al. (2004); Langley et al. (2008); Ling et al. (2009); Markovic-Housley et al. (2000); Morley et al. (2009); Reid et al. (2007); Stummeyer et al. (2005)
Proton transferring network	Asp-Ser, Asp-Tyr, Glu-Glu	GH-6, 8, 97	Honda et al. (2008); Kitamura et al. (2008); Koivula et al. (2002); Vuong and Wilson (2009)
Non-carboxylate residues	Tyr, Thr, Asn	GH-33, 34, 55, 83 (retaining), 95	Damager et al. (2008); Ferrer et al. (2005); Ishida et al. (2009); Lawrence et al. (2004); Nagae et al. (2007)
Exogenous base/nucleophile	Phosphate	GH-65, 94	Egloff et al. (2001); Hidaka et al. (2006)
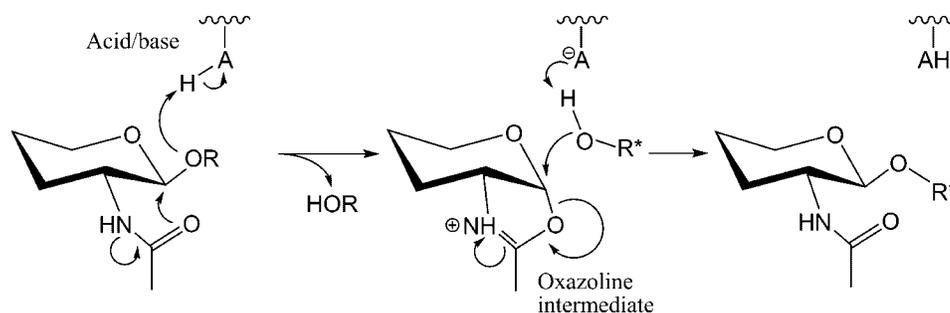
Figure 5. Substrate-assisted mechanism for retaining GHs. A 2-acetamide group in the substrate acts as a nucleophile to form an oxazoline intermediate.

has also been visualized using chemical approaches (He et al., 2010). The nucleophilicity of the acetamido group is enhanced via donation of a hydrogen bond to a suitably positioned carboxylate residue. This residue has been postulated to act as a general base to aid formation of an oxazoline intermediate in GH-84 (Cetinbas et al., 2006; Yuzwa et al., 2008) and GH-85 (Abbott et al., 2009) or, alternatively to stabilize an oxazolinium ion intermediate in GH-20 (Greig et al., 2008).

A different substrate-assisted mechanism was proposed for inverting GH-58 and GH-83 endosialidases from bacteriophage K1F (Morley et al., 2009; Stummeyer et al., 2005), where a water molecule is activated by an internal carboxylate of polysialic acid. However, biochemical proof and direct evidence from a structure of the enzyme with a ligand are necessary to confirm this mechanism, although structural analysis of the unliganded structure of a GH-58 endosialidase did not identify a catalytic base (Stummeyer et al., 2005).

Proton Transferring Network

As cellulases can play an important role in producing biofuels, their catalytic mechanism has been investigated thoroughly. A number of crystallographic and kinetic studies could not identify a catalytic base residue in many GH-6 cellulases (André et al., 2003; Koivula et al., 2002; Varrot et al., 2002; Vuong and Wilson, 2009), leading to a hypothesis that several residues act together to carry out this function by forming a proton transferring network. Molecular dynamics simulations and structural analysis of *Trichoderma reesei* cellulase Cel6A suggests Asp175 as the indirect catalytic base, which interacts with the nucleophilic water via another water molecule (Koivula et al., 2002). The nucleophilic water is held by a Ser residue and the main chain carbonyl oxygen of another Asp. This Asp-Ser proton network was further confirmed by biochemical studies in *T. fusca* cellulase Cel6B (Vuong and Wilson, 2009). As the nucleophilic water is fixed by a backbone carbonyl, single removal of the side chains could not completely eliminate

enzymatic activity, but a double mutation could (Vuong and Wilson, 2009). Structural analysis in an inverting GH-97 α-glucosidase from *Bacteroides thetaiotaomicron* showed that two Glu residues were positioned to provide base-catalyzed assistance for nucleophilic attack by a water molecule (Kitamura et al., 2008). This is similar to the two Asp residues that bind the nucleophilic water molecule in inverting GH-9 cellulases (Sakon et al., 1997). The usage of a network of several residues over a single catalytic base by cellulases shows no obvious catalytic advantage.

Another type of network was found in an inverting GH-8 exo-oligoxylanase from *Bacillus halodurans*, which is proposed to use a Hehre resynthesis-hydrolysis mechanism (Honda and Kitaoka, 2006; Honda et al., 2008), and can hydrolyze α-xylobiosyl fluoride to xylobiose in the presence of xylose or another acceptor (Fig. 6). Both Tyr198 and Asp263 hydrogen bond to the nucleophilic water molecule and mutation of Asp263 reduces the F^- releasing activity and decreases xylotriose hydrolysis, but not completely, as the Tyr still enhances the nucleophilicity of the water molecule sufficiently to break down some of the xylotriose intermediates (Honda and Kitaoka, 2006).

Utilization of Non-Carboxylate Residues

Retaining glycosidases from clan GH-E, which includes GH-33, 34, and 83 sialidases and trans-sialidases (Damager et al., 2008; Lawrence et al., 2004; Vocadlo and Davies, 2008) use a Tyr, with support from a Glu, as their nucleophile (Fig. 7). The substrate of these enzymes contains an anionic carboxylate residue at the anomeric center; therefore, nucleophilic attack by an anionic nucleophile is not favored (Watts et al., 2006). Two Asn residues in an inverting GH-95 *B. bifidum* 1,2-α-fucosidase are suggested to play critical roles in withdrawing a proton from the nucleophilic water while two acidic residues (Glu and Asp) are involved in the enhancement of water nucleophilicity (Nagae et al., 2007).

These studies indicate that non-carboxylate residues such as Tyr and Asn can function as the catalytic base/nucleophile if a neighboring carboxylate can act in concert, which
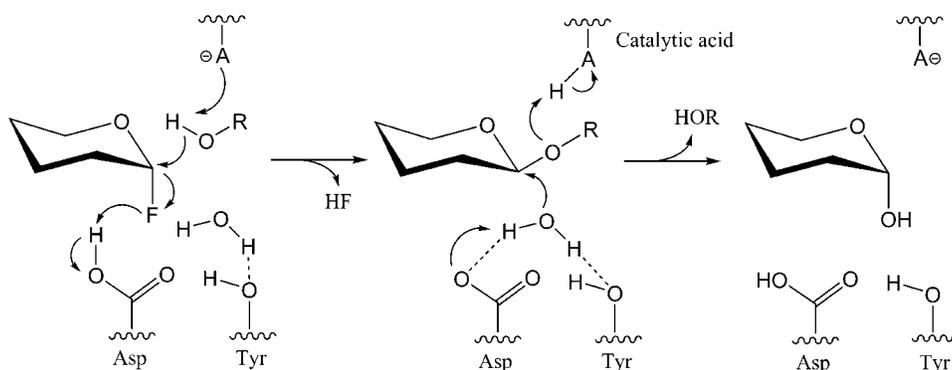
Figure 6. Asp-Tyr proton network in a GH-8 enzyme using a Hehre resynthesis-hydrolysis mechanism.

increases the options for catalytic residues. Analysis of the first structure of a GH-55 family member, *Phanerochaete chrysosporium* laminarinase did not identify a catalytic base residue (Ishida et al., 2009) even though a candidate for the nucleophilic water was found. There are no acidic residues, but the side chains of Ser204 and Gln176 as well as the main chain carbonyl oxygen of Gln146 interact with this water molecule (Ishida et al., 2009). A Thr was suggested to be the nucleophile in a *Ferroplasma acidophilum* retaining α-glucosidase, which has not been categorized into any GH family (Ferrer et al., 2005).

nicotinamide adenine dinucleotide (NAD+) as a cofactor, which is thought to oxidize the substrate at C3, thereby acidifying the proton at the C2 position; deprotonation accompanied by elimination later leads to a 1,2-unsaturated intermediate. Besides NAD+, GH-4 enzymes also require a divalent metal ion, and sometimes a reducing agent for catalysis. The metal ion is thought to stabilize the intermediate and it can be positioned by a Cys residue (Hall et al., 2009). An unusual set of retaining GH-1 enzymes, *Sinapis*
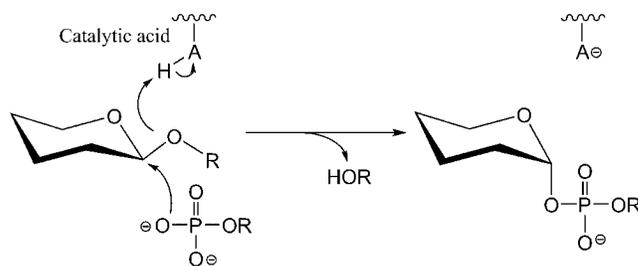
Utilization of an Exogenous Base/Nucleophile

Structural analyses suggest that inverting phosphorylases of families GH-65 (maltose phosphorylase) (Egloff et al., 2001) and GH-94 (cellobiose and chitobiose phosphorylases) (Hidaka et al., 2006) use phosphate for a direct nucleophilic attack on the anomeric center (Fig. 8). The departure of the leaving group is facilitated through partial protonation of the glycosidic oxygen by a catalytic acid residue.

Retaining GH-4 (Yip et al., 2007) and GH-109 enzymes (Liu et al., 2007) have an unusual mechanism involving

Figure 8. Phosphate as an exogenous nucleophile. This mechanism is used by inverting phosphorylases of families GH-65 and 94.
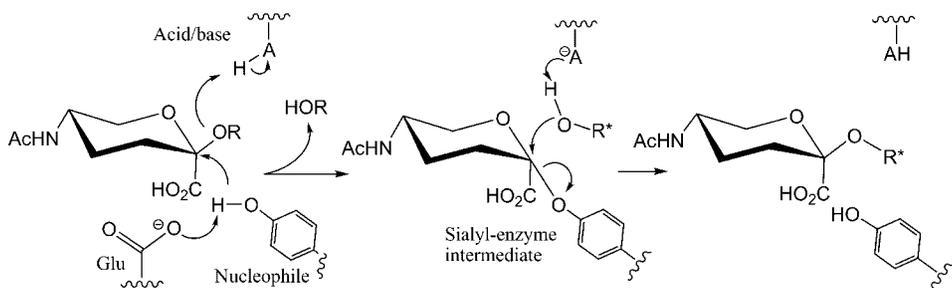

Figure 7. A non-carboxylate residue (Tyr) as a nucleophile. With support of a Glu, the Tyr attacks an anionic carboxylate residue at the anomeric center of the substrate.
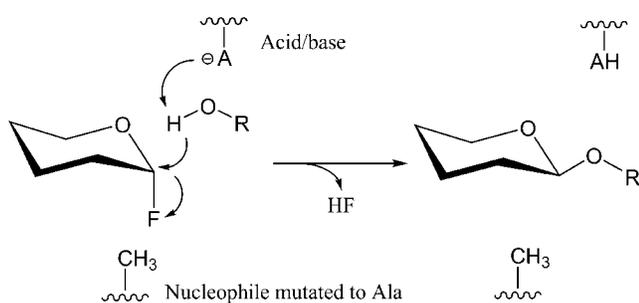
Figure 9. A glycosynthase, which is generated by mutating a glycoside hydrolase, synthesizes a glycoside from a glycosyl fluoride.

alba myrosinases (Burmeister et al., 2000) do not even require a catalytic acid/base residue, although they need a carboxylate as a nucleophile. A Gln residue positions a water molecule in the correct location without deprotonating it, this is sufficient to hydrolyze the glycosyl enzyme intermediate and release the products. However, hydrolysis is more effective when ascorbate is recruited to act as a base during the second displacement step, abstracting a proton from a water molecule and enhancing its nucleophilic attack on the anomeric center (Burmeister et al., 2000).

Although catalytic mechanisms have been proposed for 90 GH families, the catalytic base/nucleophile has not been

reported for many of them. Therefore, some GHs might actually act by yet unknown mechanisms. For instance, no potential catalytic residue or a typical catalytic center was found in the structures of two GH-61 members, although several members are proposed to have very weak glucanase activity (Harris et al., 2010; Karkehabadi et al., 2008). Some of GH-61 proteins stimulate plant cell wall degradation (Harris et al., 2010), but the way they do this is not known. It is interesting that a number of fungi have multiple GH-61 genes (Espagne et al., 2008; Tian et al., 2009), suggesting that they may enhance the hydrolysis of other polymers besides cellulose.

Removal of the Catalytic Base/Nucleophile to Create New Functions

Removal of the catalytic base/nucleophile by mutation can form a new enzyme class, glycosynthases, which catalyze the synthesis of glycosides from activated glycosyl donors, such as glycosyl fluorides. The first glycosynthase was created by Mackenzie et al. (1998) by removing the nucleophile of a retaining GH-1 β-glucosidase, producing a mutant enzyme without hydrolytic activity but with trans-glycosylation activity. Glycosyl fluoride mimics the glycosyl enzyme intermediate and acts as a glycosyl donor to an acceptor sugar, generating a glycoside with inverted anomeric stereochemistry (Fig. 9) (Mackenzie et al., 1998).
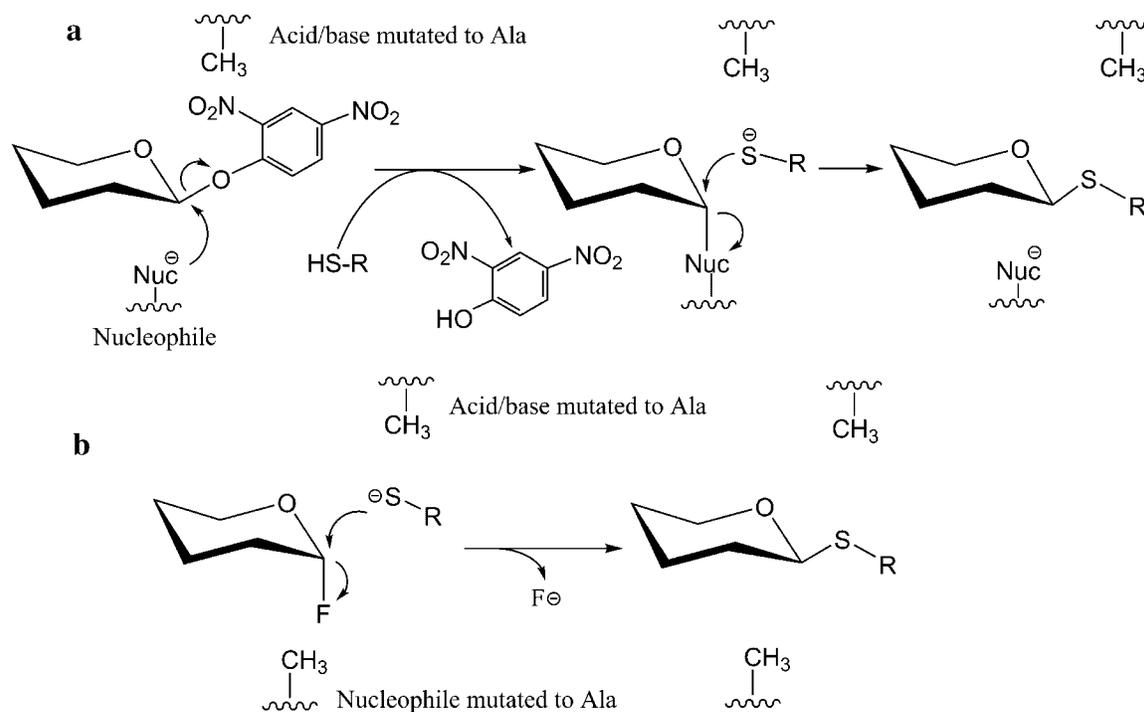

Figure 10. The synthesis of a thioglycoside by (**a**) a thioglycoligase in the presence of 2,4-dinitrophenyl glycosides and highly nucleophilic sugar thiols or (**b**) by a thioglycosynthase in the presence of glycosyl fluorides with inverted anomeric configuration and thiosugar acceptors.

Using the same principle, glycosynthases derived from several inverting glycosidases including a GH-8 β-glycosidase (Honda and Kitaoka, 2006) and a GH-95 α-glucosidase (Wada et al., 2008) were produced by mutating the catalytic base. However, more powerful glycosynthases are produced by mutation of a residue that forms hydrogen bonds with the nucleophilic water molecule (Honda et al., 2008) or a base-activating residue (Wada et al., 2008). Up to now, glycosynthases have been generated from more than 10 GH families, even from some GHs using a substrate-assisted mechanism (Umekawa et al., 2010).

Glycosynthases are gaining more attention, particularly for the synthesis of glycosides of pharmaceutical interest. For instance, glycosphingolipids show great potential as therapeutics for cancer, HIV, neurodegenerative diseases and auto-immune diseases (Hancock et al., 2009). Removal of the nucleophile of a GH-5 endoglycoceramidase II from *Rhodococcus* sp. strain M-777, coupled with directed evolution and robotic ELISA-based screening produced a double mutant enzyme with an effective glycosynthase activity for synthesizing glycosphingolipids (Hancock et al., 2009). Glycosynthases form the product in high yields and the use of glycosynthases allows regio- and stereoselective formation of glycosidic bonds (see Rakić and Withers (2009) for review). Additionally, GH-based glycosynthases might allow a wider range of substrates. The glycosynthase produced from *Humicola insolens* cellulase Cel7B by removing the nucleophile was able to effectively catalyze sugar transfer to non-sugar flavonoids, broadening the substrate specificity of the glycosynthase (Yang et al., 2007).

The general acid/base residue in retaining GHs can be mutated to generate thioglycoligases, which can synthesize thioglycosides from highly activated substrates with excellent leaving groups such as 2,4-dinitrophenyl glycosides in the presence of highly nucleophilic sugar thiols that do not require activation by the missing base residue (Rakić and Withers, 2009) (Fig. 10a). The removal of both the nucleophile and the catalytic acid/base residue of a retaining glycosidase can produce a thioglycosynthase, which catalyzes trans-glycosylations with glycosyl fluorides of inverted anomeric configuration and thiosugar acceptors (Bojarová and Kren, 2009; Jahn et al., 2004) to synthesize thioglycosides (Fig. 10b). Thioglycosides are resistant to hydrolytic cleavage by most glycosidases, thus they are of interest as metabolically stable glycoside analogues or inhibitors (Rakić and Withers, 2009).

Conclusion

The presence of these novel catalytic mechanisms in a number of GH families suggests that the diversity of catalytic residues is an approach to enhance catalytic efficiency with different substrates and this divergence requires further dividing GH members into sequence-related subfamilies based on their substrate specificity and mechanism. As key residues, the substitution of catalytic residues in glycosidases can confer completely new functions and substrate specificity, as seen in glycosynthases, thioglycoligases, and thioglycosynthases, indicating the benefit of studies of catalytic mechanisms and the power of directed mutagenesis for creating enzymes with desired properties.

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