Mechanism of catalysis by retaining β-glycosyl hydrolases

André White* and David R Rose†

Recent structural studies provide a fresh look at the catalytic mechanism of polysaccharide hydrolysis by retaining β-glycosyl hydrolases. Highlights include insights into saccharide ring distortion, both upon binding and during the course of catalysis, and evidence for the regulation of the pK_a of key catalytic residues.

Addresses
*Rosenstiel Basic Medical Sciences Research Center, Brandeis University, 315 South Street, Waltham, MA 02254-9110, USA; e-mail: awhite@auriga.rose.brandeis.edu
†Ontario Cancer Institute, and Department of Medical Biophysics, University of Toronto, 610 University Avenue, Toronto, Ontario, Canada, M5G 2M9; e-mail: drose@oci.utoronto.ca

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Abbreviations
Gal galactose
GalNAc *N*-acetyl-galactosamine
GlcNAc *N*-acetyl-glucosamine
MurNAc *N*-acetyl-muramic acid

Introduction

Retaining β-glycosyl hydrolases release their saccharide products with retention of configuration at the newly exposed anomeric center [1]. Although these enzymes may adopt a wide variety of folding motifs, their catalytic function is similar [2]. The catalytic mechanism of these enzymes was proposed some years ago to involve either a covalent glycosyl–enzyme intermediate [3] or an oxocarbenium-ion-like intermediate [4]. Evidence suggests that both states are involved, such that a covalent glycosyl–enzyme intermediate is formed and then released via oxocarbenium-ion-like transition states [5]. The catalytic nucleophile is a carboxylate sidechain, except for one example of a histidine residue [6]. Typically, a second carboxylate sidechain provides acid/base catalytic assistance, first by donating a proton to the linking oxygen of the scissile bond, and then by abstracting a proton from an incoming water molecule; this water molecule reacts with the transition-state species to form the product. Substrate cleavage is defined to occur between binding subsites −1 and +1 [7].

Recent studies from X-ray crystallography and other techniques provide structural insights into events in the catalytic mechanism of retaining β-glycosyl hydrolases. We review results that suggest that, during catalysis, the saccharide unit binding at subsite −1 adopts a 'twisted-boat' conformation, and deforms to a 'half-chair' at the transition state and then to a 'chair' at the catalytic intermediate. The final steps of the mechanism involve a reversal of this series of conformations.

Saccharide conformation on binding

Catalysis by retaining β-glycosyl hydrolases is believed to involve saccharide deformation at the enzyme's catalytic center. For lysozyme, a 'half-chair' distortion was originally proposed on the basis of early crystallographic results [4,8,9]. Recent crystallographic analyses have provided evidence of other saccharide conformations during binding and formation of the catalytic intermediate in this mechanism.

Two high-resolution crystallographic studies of hen egg-white lysozyme reveal saccharide distortion towards a 'half-chair' form, which approaches the transition-state conformation. In one study, the reaction product MurNAc-GlcNAc-MurNAc binds to the active site of lysozyme [10], whereas in a second study oligosaccharides of GlcNAc are cleaved by a mutant lysozyme[11]. In this latter report, careful refinement has revealed an inverted α-saccharide product. These results support the 'half-chair' model, although in both examples the saccharide ligand does not span the scissile linkage. Additionally, crystallographic results of the Thr26→Glu mutant of T4 lysozyme with a covalent substrate [12] suggest a 'half-chair' conformation for the catalytic intermediate. Because of its inherent non-natural modification, however, this system must be interpreted with care in the analysis of the natural catalytic intermediate.

Alternatively, the saccharide at the −1 site in retaining β-glycosyl hydrolases has been proposed to initially adopt a boat conformation on the basis of stereochemical considerations [13], although this argument is not yet generally accepted [5]. Until recently, the available experimental evidence could neither support nor exclude the possibility of a boat conformation.

There is a precedent for induced saccharide distortion on binding to proteins. For example, an unusual boat form has been observed for sialic acid (*N*-acetyl-neuraminic acid) in the influenza virus neuraminidase [14]. The carbohydrate distortion may be attributed to the carboxylic substituent at C1, which forms stabilizing electrostatic interactions with a triarginyl cluster (residues 118, 292, and 371) and advantageous packing with Tyr106. More recently, the 2.1 Å resolution crystal structure of the heat-labile enterotoxin from *Escherichia coli* complexed to the tumour marker disaccharide D-Gal-β-1,3-GalNAc reveals one sugar unit as a chair and the other as a boat conformation [15]. The geometric rearrangement of the *N*-acetylgalactosyl residue allows the attached T-antigen

protein moiety to be directed toward the outside of the binding pocket of this bacterial toxin.

Recent crystallographic evidence from a number of enzymes suggests that the saccharide at position −1 may indeed be distorted upon binding to retaining β-glycosyl hydrolases. Crystallographic analysis of the endocellulase E1 with cellotetraose suggests that the saccharide may not be bound in a relaxed chair form at subsite −1 [16••], although the nature of this deformation has not been identified. From the high-resolution crystal structure of the endoglucanase CelA from *Clostridium thermocellum* complexed to oligosaccharides, it is inferred that the saccharide unit at subsite −1 (site 'C') must undergo deformation to connect to the observed saccharide units [17•].

An abortive complex of *Serratia marcescens* chitobiase with di-*N*-acetyl-glucosamine (di-GlcNAc) has been obtained by a short ten minute incubation with its substrate [18••]. In addition to suggesting substrate assistance in catalysis by the substituent at C2 of the ligand, this crystal structure, refined to 1.9 Å resolution, shows a nonhydrolyzed disaccharide spanning the scissile bond, in which the sugar unit at position −1 forms a twisted-boat conformation. Interestingly, the presumed mechanism for this chitobiase is similar to the reverse of that proposed for the transglycosylase SLT70 [19], even though these two enzymes have different enzymatic products. A twisted-boat conformation is also observed in the crystal structure of the retaining β-glycosyl hydrolase EGI in complex with a thio-substituted nonhydrolyzable trisaccharide inhibitor [20••]. Although the resolution of this crystal structure is only 2.7 Å, the saccharide unit at position −1 is clearly constrained to adopt a twisted-boat form. The consequence of this deformation for the catalytic mechanism is that it positions the glycosidic oxygen in the proximity of the acid/base catalyst, alleviates steric hindrance between C1 and the catalytic nucleophile, and presents the aglycon leaving group in an axial position [21]. This latter aspect may have favourable stereoelectronic implications as previously predicted [13].

In contrast to β-glucose, the linking oxygen of α-glucose saccharides is inherently positioned axial in the relaxed sugar form in solution. A saccharide close to a chair conformation is observed for the nonhydrolyzable D-gluco-dihydroacarbose and acarbose inhibitors in a glucoamylase [22•]. This conformation is consistent with stereoelectronic implications for an elimination reaction [13] without requiring a deformation towards a twisted-boat conformation.

Acid/base catalyst

For a double-displacement catalytic mechanism, a side-chain provides acid/base catalytic assistance. Typically, the sidechain of this acid/base catalyst is a carboxylate located ~5.5 Å away from the catalytic nucleophile for retaining enzymes and ~9.5 Å for inverting enzymes [1,23]. In endoglucanase CelC from *Clostridium thermocellum*, the acid/base catalyst is located in a loop that is believed to fold back on the active site upon binding of the substrate [24]. This example indicates an induced-fit activation mechanism.

Because of the dual role for a general acid/base catalyst, progress through catalysis requires modulation of the ionization state of its sidechain. For example, kinetic studies with *Agrobacterium faecalis* β-glucosidase show that the pK_a of a key active-site residue changes its value by nearly three units of pH during catalysis [25]. While pK_a is a useful indicator of the ionization state of amino acids, its measurement for key catalytic residues is restricted by difficulties in distinguishing specific titration values within a protein. One means of addressing this problem is the use of 2D NMR experiments in titrating specific sidechains. In an elegant study combining 2-deoxy-2-fluoro-saccharides and ^{13}C-NMR spectroscopy on a 20 kDa xylanase from *Bacillus circulans*, the group of McIntosh [26••,27] have monitored chemical shifts of specific enzyme residue sidechains that are pH dependent. Their study reveals pK_a values of 4.6 for the catalytic nucleophile Glu78, and 6.7 for Glu172. Interestingly, the pK_a of Glu172 decreases to 4.2 in a covalent glycosyl intermediate with Glu78, consistent with a dual role of Glu172 as an acid/base catalyst. When the charge on Glu78 is abolished by forming a Gln78 mutant, a lower pK_a of 4.2 for Glu172 is observed, even in the absence of substrate. This suggests that the ionization state of the catalytic nucleophile (Glu78 for this xylanase) modulates the pK_a value of the acid/base catalyst. Alternatively, a close approach of the two key catalytic residues may explain the large shift in pK_a for the acid/base catalyst during catalysis, as proposed on the basis of the crystal structure of endocellulase E1 [16••]. Such a large decrease in the pK_a value of Glu172 is likely to be common for retaining β-glycosyl hydrolases.

Catalytic glycosyl–enzyme intermediate

Evidence from retaining β-glycosyl hydrolases other than lysozyme has generally favoured a covalent intermediate for this mechanism [5]. α-secondary deuterium kinetic isotope effects have been measured to be greater than 1.0 for two catalytic steps [5,28], which is indicative of a tetrahedral geometry at the saccharide C1 in the intermediate. In addition, Brønsted plot analyses, using a variety of substrates with different pK_a values, indicate a large degree of bond cleavage at the transition state, consistent with a covalent intermediate [25,28,29,30•]. Finally, such a covalent intermediate has been trapped by the use of 2-fluoro-glycosides with relatively reactive leaving groups [31–33].

Typically, the sidechain of either an aspartate or a glutamate fulfils the role of the catalytic nucleophile in retaining β-glycosyl hydrolases [5]. A comparison of many similar enzymes, even those with low amino acid sequence identity, may help to identify key catalytic residues, such

as for hevamine—a plant chitinase/lysozyme [34]. Frequently, more than one carboxylate sidechain is conserved for homologous β-glycosyl hydrolases; therefore, amino acid sequence analyses alone are not sufficient to identify the catalytic nucleophile. Recently, a direct strategy for identifying the catalytic nucleophile has been proposed [30•] on the basis of the formation of azide–glycoside products by mutated enzymes. In a different report, the combination of kinetic and crystallographic studies for cellobiohydrolase I from *Trichoderma reesei* and its complex with saccharides provides a useful setting to assess the importance of a catalytic trio of carboxylates [35].

In an attempt to locate the key active-site catalytic nucleophiles of retaining β-glycosyl hydrolases and their specificity of interaction, saccharide-derived epoxide compounds have often been used as labelling agents. Such epoxide compounds contain two parts: a reactive epoxide group, which forms an irreversible covalent attachment to a nucleophilic sidechain of the targeted enzyme; and a saccharide moiety, which provides specificity for binding. X-ray crystallographic studies of complexes between β-glycosyl hydrolases and such epoxide-saccharide irreversible inhibitors reveal a covalent attachment to active-site carboxylates (Table 1). Such epoxide compounds offer the promise of localizing an enzyme's active site but may not provide information about the enzyme's catalytic mechanism for three reasons. First, additional atoms mediate the attachment of the residual saccharide to the enzyme compared with the natural substrate; given the restricted space in the active site of an enzyme, these inherent additional atoms may complicate any possible conclusions on catalytic mechanism drawn from the structural interpretation. Second, the site of covalent attachment may vary depending on the epoxide compound used because of the high reactivity of these compounds. In *T. reesei endo*-1,4-xylanase II, either propyl- or pentyl-epoxide compounds bind to the putative catalytic nucleophile Glu86, whereas the butyl-derived ligands bind to the putative acid/base catalyst Glu177 [36]. This makes the assignment of particular functionalities to sidechains problematic. Third, the covalent binding of epoxides leads to irreversible complexes. Hence, the reaction mechanism followed for forming the complex is not catalytically representative for an enzyme.

In contrast to epoxide compounds, 2-fluoro-saccharide compounds can be used to trap the catalytic nucleophile in a state much closer structurally and functionally to the natural mechanism [1,33]. Upon cleavage by a retaining β-glycosidase, such a minimally modified saccharide forms a covalent species specifically with the enzyme's catalytic nucleophile; the complex formed has been shown to be catalytically competent as it slowly turns over to release the reaction product and the free enzyme [31,33,37,38•]. This strategy has been used for the β-glycanase cex-cd, which has also been extensively studied both structurally and kinetically [23,39].

Conformation of the catalytic intermediate

A glycosyl–enzyme catalytic intermediate has recently been observed in the crystal structure of the β-glycanase cex-cd with a covalently bound 2-fluoro-cellobioside substrate (Figure 1) [40••]. In this crystal structure, determined at a resolution of 1.8 Å, the saccharide in subsite −1 binds with an inverted α configuration at the anomeric carbon C1. The configuration agrees with experiments in which the intermediate is trapped

Table 1

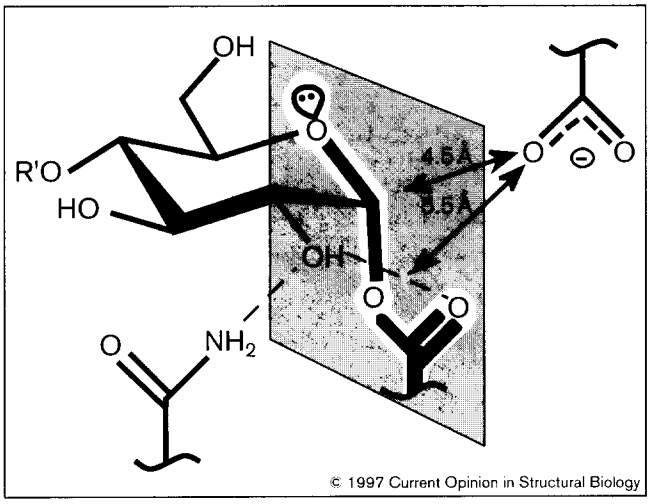
Crystal structure of β-glycosidases in complex with epoxide irreversible inhibitor affinity labels.

Epoxide-saccharide	Labelled enzyme	Labelled residue	Reference
2′,3′-epoxypropyl-β-glycoside of *N*-acetyl-D-glucosamine	Hen egg-white lysozyme*	Asp52	[49]
3,4-epoxybutyl-β-D-cellubioside	Hybrid *Bacillus* β-1,3-1,4-glucanase	Glu105	[50]
2′,3′-epoxypropyl-β-glycoside of *N,N′*-diacetylchitobiose	Human lysozyme	Asp53	[51]
2′,3′-epoxypropyl-β-glycsoside of *N*-acetyllactosamine	Human lysozyme	Asp 53	[51]
3,4-epoxybutyl-β-D-cellobioside	*Fusarium oxysporum* EGI	Glu197	[21]
3,4-epoxypropyl-β-D-xyloside	*Trichoderma reesei endo*-1,4-xylanase II	Glu86	[36]
3,4-epoxybutyl-β-D-xyloside	*Trichoderma reesei endo*-1,4-xylanase II	Glu177	[36]
3,4-epoxypentyl-β-D-xyloside	*Trichoderma reesei endo*-1,4-xylanase II	Glu86	[36]

*The structure of this complex has not been refined.

using 2-fluoro-saccharides and then analyzed using 1D ^{19}F-NMR studies [28,41]. The structure also shows that the covalently bound saccharide adopts a 'full chair' cyclic (glucopyranosyl) conformation. This latter observation is in contrast to a previously proposed controversial mechanism [42,43], in which, on the basis of stereoelectronic considerations, the retaining β-glycosyl hydrolase lysozyme would hydrolyze its substrate via an endocyclic opening of the binding saccharide.

Figure 1



Structural characteristics of the catalytic intermediate of the retaining β-1,4-glycanase cex-cd. The saccharide is shown in a full chair conformation, forming a covalent link in the inverted (α) configuration with the nucleophilic sidechain of the enzyme (lower right). This sidechain, the anomeric carbon C1, and the cyclic oxygen are nearly coplanar (shaded plane); a lone pair of the oxygen is also inferred to be coplanar. The acid/base catalyst (upper right) is positioned to activate a water molecule (not shown) for the subsequent step of the mechanism. The hydroxyl group at the C2 position of the sugar ring (shown below the ring) is positioned to accept a hydrogen bond from a conserved asparagine sidechain and the carbonyl oxygen of the nucleophilic sidechain. Remaining saccharide units are abbreviated by R'.

In the covalent complex of cex-cd [40••], the saccharide at subsite −1 binds at the *syn* position of the (now) esterified sidechain of the nucleophile Glu233. By similarity to carboxylates in solution, this *syn* position is preferred to the *anti* position in chemical reactions [44]. The orientation of the attached saccharide in cex-cd is such that its cyclic oxygen O5 is found to be nearly coplanar with the nucleophile Glu233 sidechain. As a consequence, one lone pair of electrons of O5 is inherently coplanar with the ester group of the sidechain of Glu233. The significance of this coplanarity is not known, although it may have long-range stereoelectronic implications.

In the cex-cd complex, the acid/base catalyst Glu127 is located 4.5 Å from the saccharide C1 and 5.5 Å from the nucleophile Glu233, in agreement with other retaining β-glycosyl hydrolases [1,23]. The distance of 4.5 Å is presumed to allow the positioning of a water molecule between the acid/base catalyst and the anomeric C1 in the deglycosylation step of the mechanism. In a xylanase from *Bacillus circulans*, either shortening or lengthening of the catalytic nucleophile leads to a large decrease in catalytic activity, which confirms the importance of precise positioning of the catalytic nucleophile at the active site [45]. The geometrical disposition of the acid/base catalyst relative to the nucleophile could also explain the observations on the pK_a regulation discussed above.

Previous kinetic data from other β-glycosyl hydrolases with a variety of substrates have shown that the substituent at the C2 position of the saccharide binding at subsite −1 contributes at least 8 kcal mol^{-1} of binding energy [46–48]. Inspection of the crystal structure of the cex-cd complex reveals that the fluorine substituent at C2 forms two interactions with the enzyme: it accepts a hydrogen bond from Asn126, which is a highly conserved residue among families of β-glycosyl hydrolases [24]; and it is in close proximity with the nucleophile Glu233 Oε2. Thus, a similarly positioned hydroxyl group at C2 is inferred to form a hydrogen bond with the enzyme's catalytic nucleophile, providing an explanation for the high binding energy. Indeed, this interaction between the noncovalently bonded nucleophile carbonyl and the C2 hydroxyl is seen to be 2.3 Å in a catalytically inactive mutant cex-cd complexed with a natural substrate (V Monem, RAJ Warren, SG Withers, DR Rose, unpublished data).

It is reasonable to speculate that the above structural characteristics of the catalytic intermediate observed with cex-cd are conserved for many retaining β-glycosyl hydrolases. A comparison with other reversible catalytic intermediates will better address this issue.

Conclusions: mechanism of catalysis by retaining β-glycosyl hydrolases

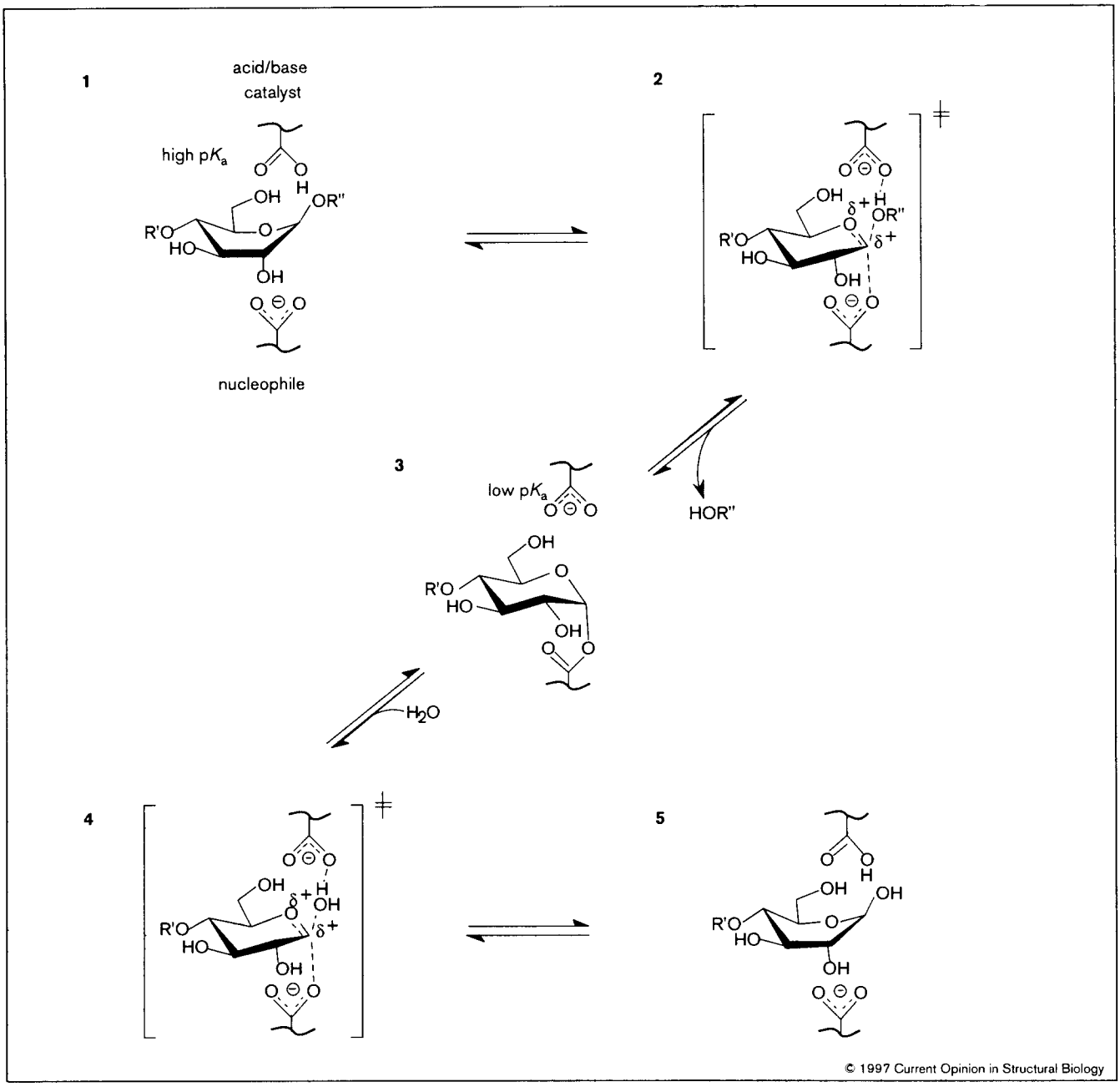
The recent evidence that we have reviewed permits us to expand the description of the catalytic mechanism as follows (Figure 2):

1. Saccharide binding at subsite −1 induces a 'twisted-boat' conformation. A general acid catalyst donates a proton to the scissile β-1,4 oxygen.

2. The β-1,4 linkage is broken, leading to the formation of a transition state with build-up of a positive charge at the anomeric carbon; typically, such a transition state develops a substantial oxocarbenium-ion-like character and resembles a 'half-chair' conformation.

3. A close approach of this ionic species to the catalytic nucleophile leads to the formation of a covalent intermediate of the inverted α configuration in a 'chair' conformation. The formation of this covalent species promotes a drop of about 2–3 units of pH in the pK_a of the acid catalyst,

Figure 2



Proposed catalytic mechanism for retaining β-1,4-glycosyl hydrolases. A covalent α-glycosyl–enzyme intermediate is formed and then hydrolyzed via oxocarbenium-ion-like transition states. For stereoelectronic considerations, the binding saccharide conformation is presumed to undergo conformational dynamics. **1.** The saccharide binds as a twisted-boat. **2.** Structure deforms to a 'half-chair' at the first transition state. **3.** Structure relaxes to a chair at the covalent intermediate. **4.** Structure deforms again to a half-chair at the second transition state. **5.** The product is a twisted-boat conformation.

which becomes a base catalyst. The aglycon is released and a water molecule diffuses into the vicinity of this base catalyst.

4. The covalent species reactivates to pass through an oxocarbenium ion-like transition state similar to the event 2 above. The base catalyst abstracts a proton from the incoming water molecule, which in turn performs a nucleophilic attack on the C1 of the residual saccharide. We expect that the formation of the product will restore the initial high pK_a of the acid/base catalyst.

5. This catalysis leads to retention of anomeric configuration at C1.

Results in the near future should confirm or modify aspects of this mechanism, including the generalization of the covalent intermediate observations to other enzyme systems and a further characterization of the transition state species.

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