Review

Mechanism of action of exo-acting α-1,4-glucan lyase: a glycoside hydrolase family 31 enzyme

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Abstract: α-1,4-Glucan lyase (GLase) performs a β-elimination reaction on α-1,4-glucans. However, GLase is different in many aspects from the polysaccharide lyase that also performs a β-elimination reaction on uronic acid containing sugars. While polysaccharide lyase mechanistically takes an anionic reaction pathway involving enolate intermediates/transition states utilizing an activated proton acidified by a uronic acid moiety, GLase does not have assisting groups and thus should overcome an enormous energy barrier to carry out catalysis. GLase achieves this goal by exploiting a similar mechanism to that of retaining glycosidases. This mechanistic aspect also can be deduced by the substantial amino acid sequence similarity of GLase to enzymes of glycoside hydrolase family 31, by which GLase is classified to glycoside hydrolase family 31. Thus, the mechanism involves the formation of a covalent glycosyl-enzyme intermediate, followed by a syn-elimination. Through the course of the reaction, highly cationic transition states are involved, as seen in the mechanism of glycosidases. GLase combines nucleophilic catalysis and general base catalysis as a smart strategy to achieve an otherwise energetically unfavorable reaction.

Key words: α-1,4-glucan lyase, retaining glycosidase, reaction mechanism, covalent intermediate, transition state, glycoside hydrolase family 31.

Abbreviations: AnFru, 1,5-anhydro-D-fructose; GH, glycoside hydrolase; GLase, α-1,4-glucan lyase; 1FGlcF, 1-fluoro-D-glucopyranosyl fluoride; 2FαGlcF, 2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride; 5FαGlcF, 5-fluoro-α-D-glucopyranosyl fluoride; 5FβIdoF, 5-fluoro-β-L-idopyranosyl fluoride; KIE, kinetic isotope effect.

Introduction

α-1,4-Glucan lyase (EC 4.2.2.13, GLase) was first discovered in *Gracilariopsis* sp. during the course of a study of the starch degrading enzymes in the red algae (Yu et al., 1999). GLase was one of two enzymes purified and initially it was thought to be an α-glucosidase. Upon identification of the product, 1,5-anhydro-D-fructose (AnFru), this enzyme was renamed as an α-1,4-glucan lyase. GLase was first purified from *Gracilariopsis* sp. in 1993 (Yu et al., 1993) and, since then, several more lyases have been purified from algae and fungi (Yu et al., 1999).

GLase acts on α-1,4-glucans such as starch and glycogen, as well as short maltooligosaccharides including maltose and maltotriose (Yu et al., 1999). GLase is an exo-acting enzyme that specifically cleaves the α-1,4-glycosidic bond in α-1,4-glucans to yield a β-elimination product from the non-reducing end sugar, leaving the reducing end sugar as free glucose (Fig. 1). The product, 1,5-anhydro-D-fructose, has been detected in several other species, while GLases have been isolated and cloned only from red algae and fungi. Thus, AnFru has also been identified in *Escherichia coli*, rat liver and human leukemic cells (Kametani et al., 1996; Suzuki et al., 1996; Shiga et al., 1999), increasing the possibility of the existence of GLase in these organisms. However, its *in vivo* role has not yet been clearly defined. In *E. coli* and rat liver, it has been found that AnFru is reduced to 1,5-anhydro-D-glucitol by a specific NADH-dependent reductase (Fig. 2). 1,5-Anhydroglucitol is possibly phosphorylated to 1,5-anhydroglucitol-6-phosphate, which is the final product of this glycogenolysis in *E. coli*. Based on these findings, a third metabolic pathway of glycogen involving GLase and reductase has been suggested (Kametani et al., 1996; Suzuki et al., 1996; Shiga et al., 1999). In fungi, a different pathway involving GLase has been suggested (Yu et al., 2004) in which AnFru is isomerized to ascopyrone P (Fig. 2), which has antibacterial and antioxidant activity, by AnFru dehydratase and as-

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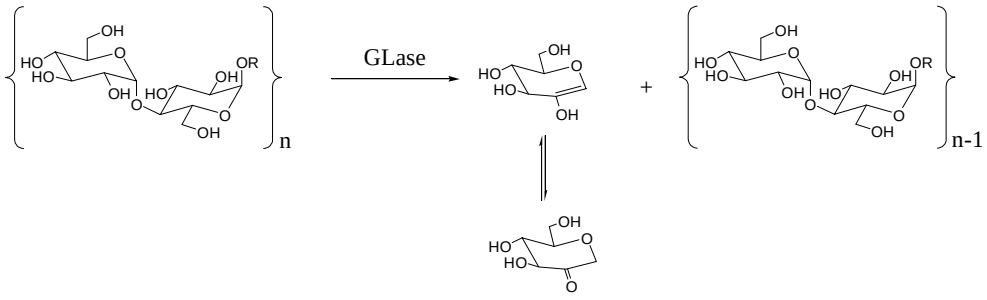

Fig. 1. Degradation of α-1,4-glucans (starch or glycogen) by the action of α-1,4-glucan lyase.

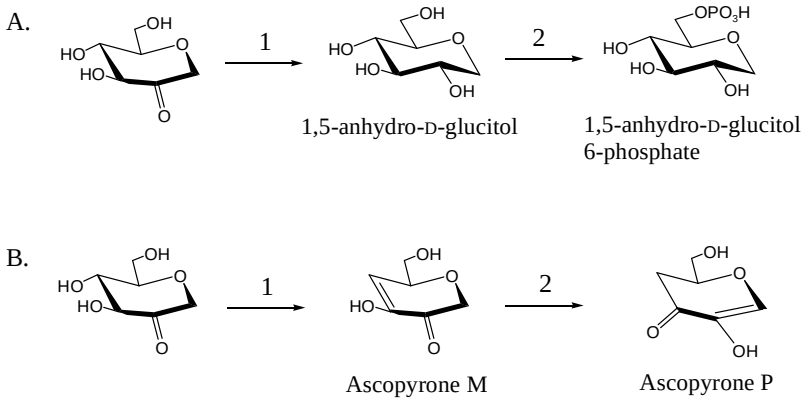

Fig. 2. *In vivo* transformation of 1,5-anhydro-D-fructose (A) in bacteria and mammals: 1. 1,5-anhydro-D-fructose reductase, 2. hexose kinase; (B) in algae and fungi: 1. dehydratase, 2. ascopyrone tautomerase.

copyrone tautomerase, suggesting a protective mechanism.

GLase is a single, non-glycosylated polypeptide chain with a molecular mass of 117-122 kDa and an optimum pH for activity of 3.8-4.1 for algal lyases and 6.5 for fungal lyases (Yu et al., 1993; 1999). GLases have a high affinity for starch, allowing the use of a starch affinity chromatography column for purification, although no starch-binding domain homologous to any currently known carbohydrate-binding domains has yet been identified (Yu et al., 1993; 1999). GLase from algae has high specificity for the α-1,4-glucan linkage and virtually no activity towards α-1,3- or α-1,6-linkages (Yu et al., 1995). Likewise, GLase does not act on other sugars such as galactosides and mannosides. This high specificity for the α-1,4-glucan linkage is consistent with the existence of several subsites, leading to higher activity for glucan polymers and maltooligosaccharides such as starch, glycogen, maltose and maltotriose than for synthetic monosaccharide substrates such as *p*-nitrophenyl α-D-glucopyranoside (Yu et al., 1995; Yoshinaga et al., 1999).

GLase catalyzes formally a β-elimination as do polysaccharide lyses and, thus, GLase itself is a polysaccharide lyase. However, GLase is different from other polysaccharide lyases in many aspects. First of all, while substrates for polysaccharide lyases are uronic acid-containing polysaccharides, those for GLases are simple α-1,4-glucans. Secondly, GLase cleaves the glycosidic bond between the anomeric carbon of the non-reducing

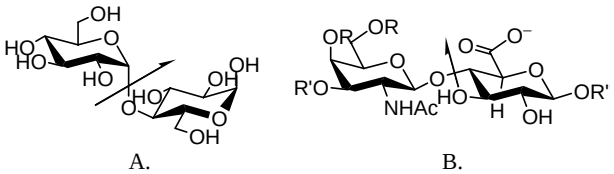

Fig. 3. Scissile bonds of substrates for lyases. Scissile bonds are designated by arrows: (A) α-1,4-glucan lyase; (B) polysaccharide lyase.

sugar and the glycosidic oxygen while polysaccharide lyases cleave the bond between the glycosidic oxygen and C4 of the reducing sugar (Fig. 3). The location of the double bond generated by the reaction is also different. GLase produces a double bond between C1 and C2 of the non-reducing sugar whereas polysaccharide lyase creates unsaturation between C4 and C5 of the reducing sugar.

Mechanistically, GLase is very unusual. While the substrates of polysaccharide lyase contain a uronic acid moiety that can activate the proton abstracted during the reaction (Gacesa, 1987; Rye & Withers, 2002), substrates of GLase have no functional groups to activate the proton on C2 which is abstracted. In addition, it seems that GLase activity does not depend on redox chemistry, which might activate the proton *in situ,* since no NAD, NADP or metal dependence have been found. It is therefore intriguing to understand how GLase can overcome the enormous activation barrier in-

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A. Signature Region 1                                        *
Barley High pI α-glucosidase                          432  DGLWIDMNEISNF  444
Sugar beet α-glucosidase                              464  DGIWIDMNEASNF  476
Human isomaltase                                      500  DGLWIDMNEVSSF  512
Human sucrase                                        1389  DGLWIDMNEPSSF 1401
Human lysosomal α-glucosidase                         513  DGMWIDMNEPSNF  525
Schizosaccharomyces pombe α-glucosidase               476  SGIWTDMNEPSSF  489
Aspergillus niger α-glucosidase, P2 subunit           219  DGVWYDMSEVSSF  231
Garcilariopsis sp. α-1,4-glucan lyase                 548  DFVWQDMTVPAMM  560
Morchella costata α-1,4-glucan lyase                  543  EFVWQDMTTPAIH  555
Arabidopsis thaliana α-xylosidase (XYL1)              435  DGLWIDMNEVSNF  447
Tropaeolum majus α-xylosidase                         454  DGLWIDMNEDLEF  466
Pinus pinaster α-xylosidase                           433  DGLWIDMNEISNF  445
Lactobacillus pentosus α-xylosidase (XylQ)            409  DSFKTDFGER---  418
Escherichia coli K12 ORF (yicI)                       411  DCFKTDFGER---  420
Sulfolobus solfataricus α-xylosidase (XylS)           348  DAYWLDASEP---  357

B. Signature Region 2
Barley High pI α-glucosidase                  561  GADICGFNG----NTTEELCGRWIQLGAFYPFSR  590
Sugar beet α-glucosidase                      595  GADICGFAE----STTEELCCRWIQLGAFYPFSR  624
Human isomaltase                              631  GADICGFVA----ETTEELCRRWMQLGAFYPFSR  660
Human sucrase                                1527  GADICGFFN----NSEYHLCTRWMQLGAFYPYSR 1556
Human lysosomal α-glucosidase                 643  GADVCGFLG----NTSEELCVRWTQLGAFYPFMR  672
Schizosaccharomyces pombe α-glucosidase       674  GADVCGFLG----DSDEELCSRWMANGAFSPFYR  703
Aspergillus niger α-glucosidase, P2 subunit   421  GADTCGFNG----NSDEELCNRWMQLSAFFPFYR  450
Garcilariopsis sp. α-1,4-glucan lyase         692  GSDIGGFTS( 8)PCTGDLMVRYVQAGCLLPWFR  729
Morchella costata α-1,4-glucan lyase          662  GSDTGGFEP(10)YCSPELLIRWYTGSFLLPWLR  701
Arabidopsis thaliana α-xylosidase (XYL1)      590  GSDICGFYP----QPTEELCNRWIEVGAFYPFSR  619
Tropaeolum majus α-xylosidase                 609  GSDICGFYP----GPTEELCNRWIEVGAFYPFSR  638
Pinus pinaster α-xylosidase                   597  GADICGFYP----DTTEELCGRWIQLGAFYPFSR  626
Lactobacillus pentosus α-xylosidase (XylQ)    508  SHDIGGFED( 3)TPTADLYKRWSQFGLLSSHSR  540
Escherichia coli K12 ORF, yicI                509  SHDIGGFEN----TAPAHVYKRWCAFGLLSSHSR  538
Sulfolobus solfataricus α-xylosidase (XylS)   455  TTDTGGFFS( 5)KAYAEIFVRWFQWSTFCPILR  489
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Fig. 4. Partial multiple sequence alignment of α-glucosidases, α-1,4-glucan lyases and α-xylosidases of GH family 31. A. Signature Region 1; B. Signature Region 2. Proposed catalytic nucleophile is in bold character and indicated by an asterisk (*). Accession numbers: barley high pI α-glucosidase (GenBank AF118226), sugar beet α-glucosidase (SwissProt O04931), human sucrase-isomaltase (SwissProt P14410), human lysosomal α-glucosidase (SwissProt P10235), *Schizosaccharomyces pombe* α-glucosidase (GenBank AB045751), *Aspergillus niger* α-glucosidase, P2 subunit (SwissProt P56526), *Gracilariopsis* sp. α-1,4-glucan lyase (SwissProt P81676), *Morchella costata* α-1,4-glucan lyase (SwissProt P81696), *Arabidopsis thaliana* α-xylosidase (GenBank AAD37363), *Tropaeolum majus* α-xylosidase (GenBank CAA10362), *Pinus pinaster* α-xylosidase (GenBank AF448201), *Lactobacillus pentosus* α-xylosidase (GenBank AAC62251), *Escherichia coli* K12 ORF yicI (SwissProt P31434), *Sulfolobus solfataricus* α-xylosidase (SwissProt Q9P999).

volved in abstracting a non-acidic, non-activated proton.

Amino acid sequence alignments also reveal interesting features (Yu et al., 1999). The two subfamilies of sequenced GLases, algal and fungal enzymes share no amino acid sequence similarity with any known polysaccharide lyases. Instead, GLases exhibit 23–28% sequence similarity with α-glucosidases of glycoside hydrolase (GH) family 31, including two signature regions (Fig. 4). Therefore, the two subfamilies (algal and fungal) of GLases are assigned to GH family 31 and posted as such on the CAZY web site (http://afmb.cnrs-mrs.fr/CAZY) on the basis of amino acid sequence similarity despite the difference in reactions catalyzed. GH family 31 is a big glycosidase family that contains mostly α-glycosidases, including such important enzymes as the human lysosomal α-glucosidase whose deficiency results in Pompe's disease, the endoplasmic reticulum glucosidase II that plays a key role in glycoprotein processing and folding, and the digestive enzyme sucrase-isomaltase that is the target of inhibition by the anti-diabetes drugs acarbose and miglitol. These are retaining α-glycosidases whose mechanism involves the formation and hydrolysis of a covalent glycosyl-enzyme intermediate via highly positively charged oxocarbenium ion-like transition states. Unlike GH family 13 α-glycosidases for which three-dimensional structures have been known for some 20 years, the structure of a GH family 31 enzyme only recently became available (Lovering et al., 2005).

In this review, the discussion will focus on mechanistic aspects of this unusual enzyme and relevant recent achievements will be presented.

Anionic vs. cationic mechanism: enzymatic eliminations in nature

The reaction catalyzed by α-1,4-glucan lyase is an overall *anti*-elimination. There are many enzymes catalyzing either anti- or syn-elimination reactions in nature and most of them carry out this reaction by exploiting the presence of an activated proton to be abstracted that is acidified by an adjacent carboxylic acid group. Polysaccharide lyase is one such enzyme. The general base residue that is responsible for abstracting the proton is often an oxygen containing base in polysaccharide lyases. Since the proton is transferred from a less electronegative carbon atom to a more electronegative oxygen atom, the aid of some other acidifying group is absolutely necessary for the catalysis. The formation of this oxyanion intermediate is followed by β-elimination (Rye & Withers, 2002). This strategy is adopted by most enzymes catalyzing elimination reactions. For another example, the enolase superfamily is among the largest of the groups of enzymes performing a β-elimination reaction. Abstraction of a proton activated

by an adjacent carboxylic acid group, generates an anionic enolate intermediate which subsequently undergoes either *anti*- or *syn*-elimination (Babbit & Gerlt, 1997). Dehydratases in the N-acetylneuraminate lyase superfamily utilize a slightly different, but identical in principle, strategy in activating the proton. The β-elimination is initiated by abstracting a proton that is activated by a protonated Schiff base (Babbit & Gerlt, 1997). Crotonase also catalyzes a β-elimination involving an oxyanion intermediate, though in this case the α-proton of a thioester is abstracted (Babbit et al., 1992). These examples, without exception, utilize an anionic mechanism (E1cB) with the aid of an electron withdrawing functionality in the substrate. There have been only very few examples of mechanisms of enzymatic elimination reactions different from the E1cB type (Smar et al., 1991; Morgan et al., 1997). In the case of GLase, however, it is obvious that this more common anionic reaction pathway is unlikely, since the α-1,4-glucan substrates do not contain an electron-withdrawing functional group that can activate the proton. Further, GLase shares sequence similarity with retaining glycosidases that are known for cationic mechanisms involving oxocarbenium ion-like transition state. This would argue for a cationic transition state rather than an E1cB type mechanism. As the overall reaction is an *anti*-elimination, the reaction could proceed via direct E1 or E2 *anti*-elimination reactions. Alternatively, it is reasonable to postulate a mechanism analogous to the double displacement mechanism of retaining α-glucosidases, involving the formation of a covalent glycosyl-enzyme intermediate via an oxocarbenium ion like transition state, followed by a *syn*-elimination in the case of GLase. In the first step, a combination of nucleophilic attack and general acid catalysis would operate to form a covalent glycosyl-enzyme intermediate, thereby providing a better leaving group for the subsequent β-elimination reaction via a cationic transition state.

Active site environments

Retaining glycosidases contain two carboxylic acids in the active site, acting as acid/base catalyst and as nucleophile/leaving group. Reflecting this, a bell-shaped pH dependent activity has been observed for most retaining glycosidases, indicating a carboxylate group, acting as a nucleophile and a protonated carboxylic acid, acting as a general acid. Since GLase has similar structural features, as suggested by the invariant aspartic acid residues of GH family 31, a similar dependence of the activity on pH might be expected. Values of $k_{\mathrm{cat}}/K_{\mathrm{m}}$ for 2,4-dinitrophenyl α-D-glucopyranoside were measured at a series of pH values and the resulting pH-activity profile proved to be indeed a classical bell-shaped curve suggesting that there are at least two essential ionizable groups (Fig. 5) (Lee et al., 2003). Consistent with the initial expectation, the

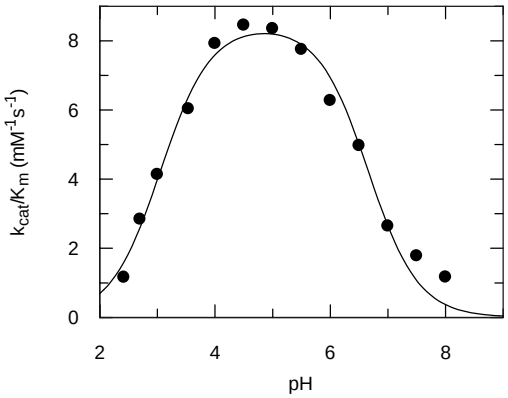


Fig. 5. pH-dependent degradation of 2,4DNPαGlc by GLase (excerpted from Lee et al., 2003).

pH optimum (4.8) and the pH-dependence of $k_{\mathrm{cat}}/K_{\mathrm{m}}$ ($\mathrm{p}K_{a1}$ = 3.1, $\mathrm{p}K_{a2}$ = 6.7) for GLase were very similar to those of the GH family 31 *Aspergillus niger* α-glucosidase which has a pH optimum of 4.5, and $\mathrm{p}K_{a1}$ = 3.2 and $\mathrm{p}K_{a2}$ = 6.4 (Kimura et al., 1997). Also, carboxylic acid groups in the active site are suggested by the finding that a water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, inactivates GLase (Yoshinaga et al., 1999; Nyvall et al., 2000). This is further supported by the inhibition by 1-deoxynojirimycin (Yu et al., 1995; Nyvall et al., 2000; Lee et al., 2003). Inhibition of glycosidases by this compound may be considered as evidence for oxocarbenium ion character of the transition state and of anionic groups in the active site.

The active site of GLase was suggested to have subsites based on values of k_{cat} and K_{m} for a series of maltooligosaccharides (Yoshinaga et al., 1999). The k_{cat} values were virtually identical for all substrates whereas K_{m} values decreased as the number of glucose units increased up to four (maltotetraose), and beyond that, remained identical. This may indicate the existence of four subsites which have favorable binding interactions with substrates. It is therefore likely that the subsite substrate specificity of GLases is very similar to that of type III α-glucosidases (Frandsen & Svensson, 1998).

A covalent intermediate and two-step mechanism

The similarity of the active site environment of GLase to that of retaining α-glucosidases, in combination with other previously described evidence, may indicate the close kinship of GLase with GH family 31. The most direct approach to prove the striking similarity between the two classes of enzymes was to search for the intermediate. The obvious reagents for this task are fluorosugars that have been employed for this task on retaining glycosidases (Mosi & Withers, 2002; Wicki et al., 2002). The highly electronegative fluorine adja-

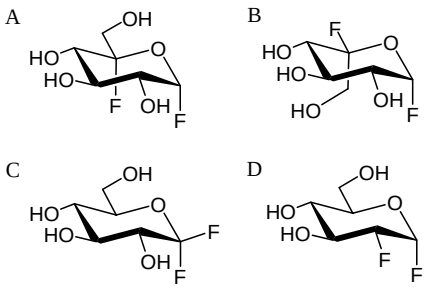


Fig. 6. Difluorosugars designed as mechanism based reagents. (A) 5-fluoro-α-D-glucopyranosyl fluoride; (B) 5-fluoro-β-L-idopyranosyl fluoride; (C) 1-fluoro-D-glucopyranosyl fluoride; (D) 2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride.

cent to the anomeric center destabilizes the oxocarbenium ion-like transition states, slowing both the formation and the hydrolysis of the intermediate. However, the presence of a good leaving group, fluoride, at the anomeric center ensures that the formation of the intermediate (glycosylation) is faster than its hydrolysis (deglycosylation). As a result, the glycosyl-enzyme intermediate will accumulate, in some cases resulting in inactivation of the enzyme if turnover is very slow. The mechanism-based inactivators (Fig. 6), 5-fluoro-α-D-glucopyranosyl fluoride (5FαGlcF) and 5-fluoro-β-L-idopyranosyl fluoride (5FβIdoF), have been used to trap covalent glycosyl-enzyme intermediates on retaining α-glucosidases (McCarter & Withers, 1996a,b; Lee et al., 2001), including the trapping of the covalent glycosyl-enzyme intermediate on a GH family 31 α-glucosidase (Lee et al., 2001). In many cases, epimers of the parent fluorosugars inverted at C5 have turned out to be more effective trapping reagents (Howard et al., 1998; Numao et al., 2000) and indeed 5FβIdoF is a more potent α-glucosidase inactivator (McCarter & Withers, 1996b). Other fluorosugars such as 2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride (2FαGlcF) and 1-fluoro-D-glucopyranosyl fluoride (1FGlcF) have also proved useful, with 2FαGlcF having been shown to act as a slow substrate rather than an inactivator for α-glucosidases (Mosi & Withers, 2002). 1FGlcF has been developed as a probe for the development of positive charge during glycoside hydrolysis and acted as a slow substrate for both α- and β-glycosidases (Konstantinidis & Sinnott, 1991).

Some interesting results were reported with GLase. While 2FαGlcF and 1FGlcF did not inactivate and 5FαGlcF only showed a small amount of inhibition, 5FβIdoF was shown to inactivate GLase in a time and concentration dependent manner (Lee et al., 2002; 2003). 2FαGlcF, 1FGlcF and 5FαGlcF are thought to act as slow substrates with substantially reduced rates compared to that of the parent compound, α-D-glucopyranosyl fluoride. The implication of the reduced rates for these substrates was discussed thoroughly (Lee et al., 2003) and will be presented later in this review.

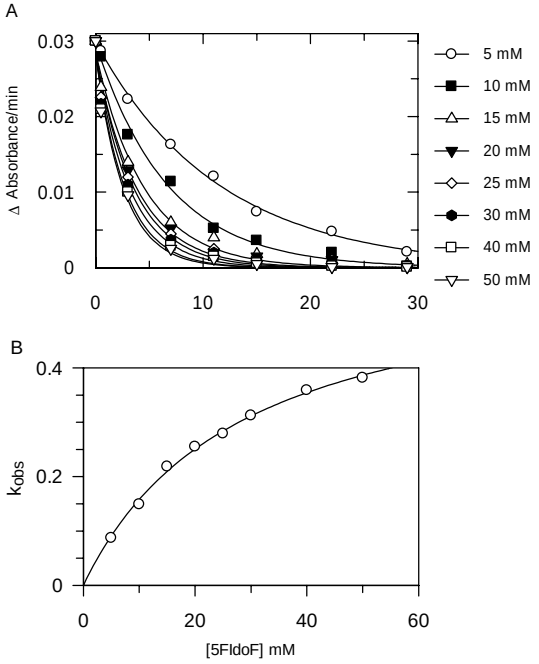


Fig. 7. Inactivation of GLase by 5FβIdoF. (A) Residual activity at each time interval; (B) replot of apparent rate constants from A.

Direct evidence for the formation of a covalent glycosyl-enzyme intermediate came from inactivation studies with 5FβIdoF (Lee et al., 2002). It was reported that the inactivation afforded by this compound followed pseudo first-order kinetics (Fig. 7). Values of pseudo first order kinetic constants, k_{obs}, at various concentrations of 5FβIdoF, fitted to the equation, $k_i = k_{obs}/(K_i + [I])$, yielded an inactivation rate constant k_i of 0.61 ± 0.02 min^{-1} (or 0.010 s^{-1}) and an inactivator dissociation constant, K_i, of 28.3 ± 2.1 mM. A competitive inhibitor, acarbose, afforded protection from inactivation by 5FβIdoF, indicating that inactivation occurs at the active site (Fig. 8A). The catalytic competence of the trapped intermediate and the relevance of the inactivation process were assessed by monitoring reactivation. In most cases of retaining glycosidases, accumulated glycosyl-enzyme intermediates have been shown to be kinetically competent and to turn over upon removal of excess inactivator. Likewise, GLase, pre-inactivated through incubation with 5FβIdoF, was shown to be reactivated slowly upon removal of the excess inactivator. Slow turnover followed first order kinetics with a rate constant of $k_{react.} = 0.036$ min^{-1} or 6.0×10^{-4} s^{-1} (Fig. 8B). These indicate that all events occur at the active site of GLase and the reactivation of inactivated GLase through turnover of the covalent intermediate is clear evidence for the competence of the intermediate.

These results provide substantial support for the existence of a covalent intermediate at the active site of the GLase. Further, the intermediate formed was isolated and analyzed (Lee et al., 2002), as is done with

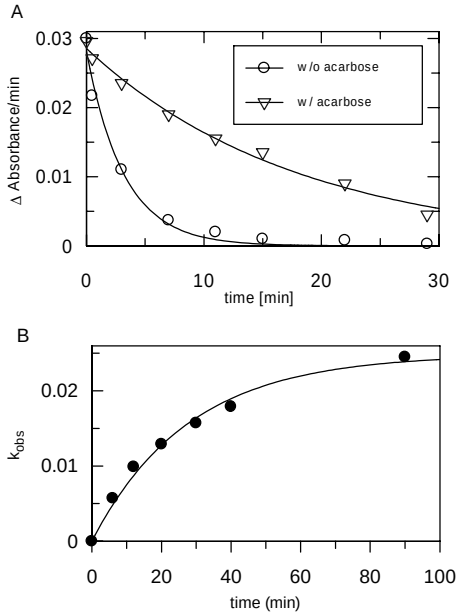


Fig. 8. (A) Protection from inactivation in the presence of 0.2 μM acarbose ($K_i = 0.02$ μM) at 30 mM 5FidoF; o without acarbose; ∇ with acarbose. (B) Reactivation of inactivated α-1,4-glucan lyase upon removal of excess 5FIdoF.

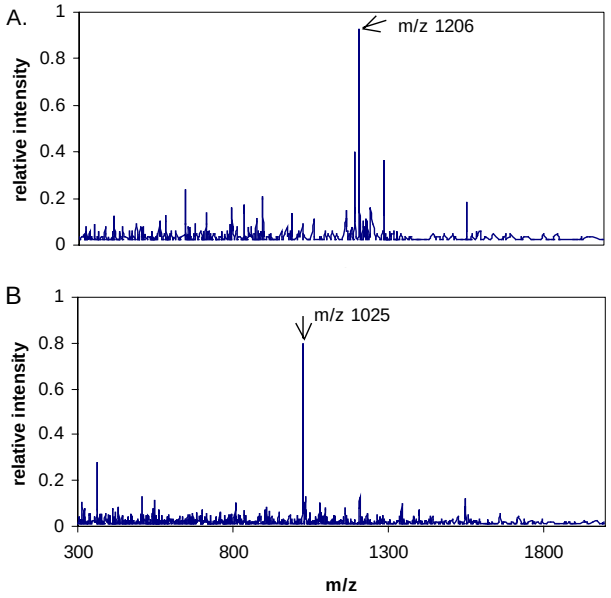


Fig. 9. Detection of 5FIdo-labeled peptide by comparative mapping. Mass spectrum (z = 1) of the peptic digest from the labeled enzyme (A) taken at 40.48 min and from the unlabeled enzyme (B) taken at 41.55 min.

glycosidases. Fully inactivated GLase, along with a control sample, was subjected to peptic digestion followed by LC/ESI MS comparative mapping. The masses of the peptides under each peak from the labeled and unlabeled enzymes were compared. A peptide fragment corresponding to m/z 1206 was detected only in the inactivated sample while a fragment of m/z 1025, lower in mass by the amount expected for the 5-fluoro-β-L-idosyl moiety (m/z = 181), was detected in the control sample (Fig. 9). This strongly suggests that the fragment of m/z 1206 is the active site peptide labeled by 5FβIdoF and therefore contains the catalytic nucleophile. This fragment was isolated and sequenced by tandem mass spectrometry (Fig. 10).

The daughter ion spectrum (Fig. 10B) reveals a fragment corresponding to mass 1025 (mass difference by m/z = 181 from the parent ion) arising from the peptide without label. Although interpretation of the rest of the fragmentation pattern was not fruitful, an excellent fragmentation pattern was observed in the daughter ion spectrum of the species of m/z 1025 generated by orifice fragmentation (Fig. 10A). This readily yielded a sequence of FVWQDMTV. The spectrum also reveals three labeled peptide fragments of m/z 645, 989 and 1089 which are consistent with peptides DMTV, FVWQDM and FVWQDMT, each bearing the 5FβIdo moiety. This strongly suggests that Asp553 is the catalytic nucleophile, which is absolutely conserved in all GH family 31 enzymes. The equivalent residue has been proposed previously as the catalytic nucleophile in family 31 α-glucosidases (Frandsen & Svensson, 1998; Lee et al., 2001).

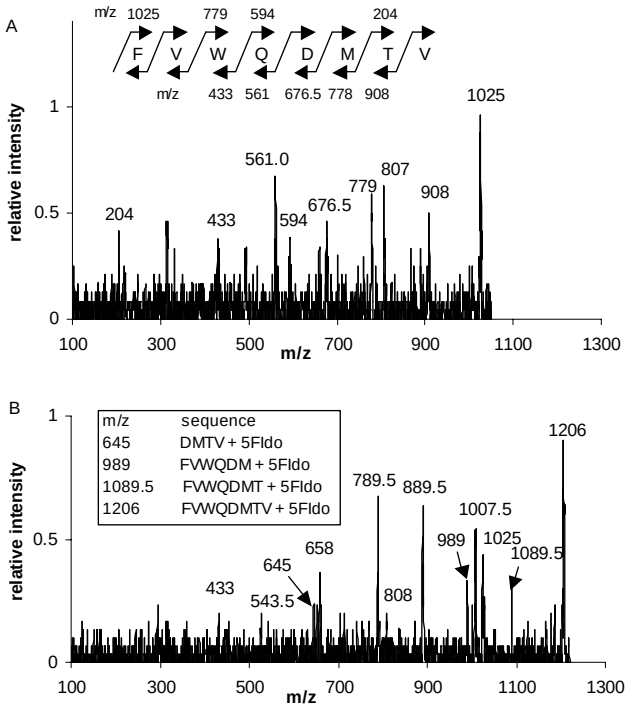


Fig. 10. ESI MS/MS daughter ion spectrum along with interpretation. (A) Daughter ion spectrum (z = 1) of m/z 1025 peak from spectrum B; (B) daughter ion spectrum of m/z 1206 peak from labeled peptide (excerpted from Lee et al., 2002).

The inactivation by fluorosugars implies not only a mechanism involving a covalent intermediate but also positively charged transition states. Thus, the formation and elimination of the covalent intermediate on GLase are likely to involve cationic transition states.

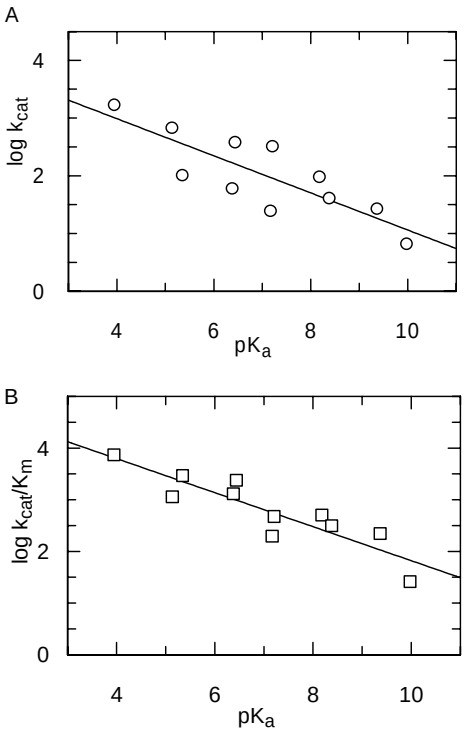


Fig. 11. Brønsted plot constructed from data of Table 3 showing the relationship of the rate of cleavage of a series of aryl glucosides with the pK_a of the corresponding phenol. (A) log k_{cat} versus pK_a; (B) log k_{cat}/K_m versus pK_a (Lee et al., 2003).

Anatomy of the mechanism of GLase

Brønsted relationships

Brønsted relationships have proved to be useful tools to probe the transition state structures of enzymatic reactions, providing information on the structure of the relevant transition state (Kempton & Withers, 1992; Li et al., 2001; Vocadlo et al., 2002). Brønsted relationships for cleavage of aryl glucosides by GLase have shown that both k_{cat} and k_{cat}/K_m are only moderately sensitive to changes in leaving group pK_a value, with k_{cat} values changing only 250 fold from the most to the least reactive substrates ($\Delta pK_a = 6$) (Lee et al., 2003). The plots of log k_{cat} and log k_{cat}/K_m vs. pK_a exhibit a significant level of scatter ($R = 0.83$ and 0.90, respectively), but clearly reveal a dependence, albeit shallow ($\beta_{lg} = -0.32$ and -0.33, respectively), of rate on leaving group ability (Fig. 11). The similarity in these slopes suggests that the same step is rate-limiting in the two cases and the clear dependency on pK_a indicates that this step involves breakage of the aryl glycoside bond. This is reinforced by the fact that k_{cat}/K_m reports on the first irreversible step, which has generally proved to be the formation of the glycosyl-enzyme for retaining glycosidases. The low β_{lg} value indicates that relatively little negative charge is present on the glycosidic oxygen at the transition state. Thus either there is very little glycosidic bond cleavage at the transition state or considerable proton donation has occurred. The former interpretation is rendered unlikely by the inactivation afforded by a fluorosugar, 5FβIdoF, and by the measurement of large α-deuterium kinetic isotope effects (discussed later) both of which indicate substantial oxocarbenium ion character thus, substantial bond cleavage. Early proton donation, thereby reducing net negative charge, therefore seems likely, as suggested for the first step of the sucrase-isomaltase complex of the same gene family, GH family 31 (Cogoli & Semenza, 1975) where similarly low, but clear, dependence of rates on the pK_a values of leaving groups was observed along with a large α-deuterium kinetic isotope effect. Hammet-Hansch analysis for both enzymes (sucrase and isomaltase) yielded a low ρ value, which was very close to that measured for acid-catalyzed hydrolysis of aryl glucosides in which the glycosidic oxygen is almost fully protonated with the glycosidic bond being largely broken at the transition state. This led to the suggestion that the transition state would mimic the features of the acid-catalyzed hydrolysis reaction. Early protonation accompanying bond breakage has been seen previously for the first step of retaining glycosidases: indeed similarly low β_{lg} values have been seen with the *Cellulomonas fimi* exoglycanase and interpreted likewise (Tull & Withers, 1994).

Fluorosugars as slow substrates

As noted earlier three of the fluorosugars, 1FGlcF, 2FαGlcF and 5FαGlcF, are in fact slow substrates and kinetic parameters measured using a fluoride electrode gave useful insights. Table 1 summarizes results of these kinetic analyses (Lee et al., 2002; 2003). 1FGlcF and 2FαGlcF were reported to bind poorly with high K_m values that could not be accurately measured. Consequently only values of k_{cat}/K_m were measured for these two compounds, though values of k_{cat} and K_m could be measured for 5FαGlcF. $\Delta\Delta G^{\ddagger}$ values for all three difluorides (1FGlcF, 2FαGlcF and 5FαGlcF) were calculated to reflect the change in the free energy of activation for the first irreversible step (k_{cat}/K_m) compared to the parent sugar, αGlcF using the equation $\Delta\Delta G^{\ddagger} = RT\ln[(k_{cat}/K_m)_F/(k_{cat}/K_m)_{F2}]$ where the subscript F indicates the value for the monofluoride and F2 for the difluorides.

One approach to probe charge development involves measurement of the effects of fluorine substitution adjacent to the reaction center upon reaction rates. The effects of substitution of a second fluorine at C-1, C-2 and C-5 upon rates of cleavage of αGlcF were indeed explored. Substitution at C-1 and C-5 involves replacement of H by F, which may cause very minor steric penalties, but does not remove any potentially important hydrogen bonding interactions, as is possibly the case at C-2. Further, substitution at C-2 and C-5 is adjacent to the developing oxocarbenium ion, whereas substitution at C-1 is directly on the carbocation. Previous non-enzymatic solvolysis studies with 2-deoxy-2-fluoro sugar derivatives have revealed

Table 1. Michaelis-Menten parameters for the cleavage of fluorosugars by *Gracilariopsis* α-1,4-glucan lyase (Lee et al., 2003).

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}M^{-1}$)	$\Delta\Delta G^{\ddagger}$ (kJ/mol)
1FGlcF			13.8	18.1
2FαGlcF			3.81	21.1
5FαGlcF	0.131	10.7	12.2	18.4
αGlcF	505	27.9	1.81×10^4	

an approximately 60-fold rate reduction as a consequence of substitution at the 2-position (Withers et al., 1986). Effects of substitution right at the cationic center are less predictable. Indeed a stabilizing effect by lone pairs on fluorine substituted directly on carbocations has been seen (Blint et al., 1974), though studies of spontaneous solvolysis of 1FGlcF have revealed substantial rate reductions corresponding to an increase of free energy of activation ($\Delta\Delta G^{\ddagger}$) of 18.5 $kJmol^{-1}$ (Konstantinidis & Sinnott, 1991). The difference in behavior in these two cases presumably resides in the oxocarbenium ion character present in this case: lone pair donation by the oxygen likely dominates any possible donation by fluorine, leaving only the destabilizing inductive effect of the fluorine. Interestingly, $\Delta\Delta G^{\ddagger}$ values for all three substitutions on the GLase's enzymatic reaction were approximately 20 $kJmol^{-1}$, thus are very similar to that seen for spontaneous solvolysis. Since the non-enzymatic process has been shown to involve substantial carbocationic character (Sinnott& Jencks, 1980; Banait & Jencks, 1991), a similar transition state is predicted for the enzymatic process. Therefore, since k_{cat}/K_m reflects the first irreversible step, the changes in the free energy of activation reflect the destabilization of an oxocarbenium ion-like transition state of the first, glycosylation, step. Inspection of the data in Table 1 also reveals that the K_m value for 5FαGlcF is 3-fold lower than that for αGlcF, despite potential steric hindrance from the fluorine at C5. This relatively high affinity is most likely due to the second step being slowed substantially more than the first by the fluorine substitution, with an accumulation of intermediate resulting. This phenomenon leads to a lowering of the K_m value when the second step is clearly rate-limiting. The expression for K_m for a two-step enzymatic reaction is as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E\text{-}S \xrightarrow{k_3} E + P$$

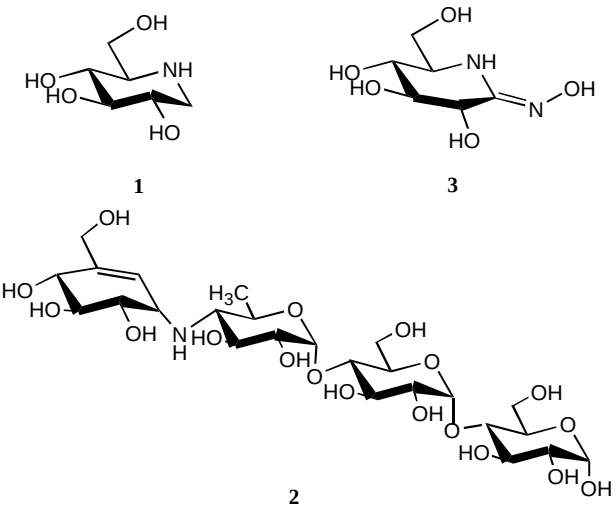
ES: Michaelis complex
E-S: covalent intermediate

$$K_m = \left(\frac{k_{-1} + k_2}{k1}\right)\left(\frac{k_3}{k_2 + k_3}\right)$$

where k_2 is the rate constant for the first step and k_3 that for the second step. If the second step is rate limiting, then $k_2 \gg k_3$ and the second factor will be reduced to k_3/k_2 ($\ll 1$) which would yield a lower K_m value. Similar cases have been observed in several glycosidases. K_m values for 5FαGlcF were too small to measure with a GH family 31 α-glucosidase from *A. niger* (Lee et al., 2001). Similarly low K_m values were proposed for yeast α-glucosidase (GH family 13) and *Coffea arabica* α-galactosidase (GH family 27) (McCarter & Withers, 1996a; Ly et al., 2000).

Table 2. The structure of inhibitors and K_i values for *Gracilariopsis* α-1,4-glucan lyase (Lee et al., 2003).

Inhibitors	K_i (μM)
1-Deoxynojirimycin (**1**)	0.13 ± 0.001
Acarbose (**2**)	0.020 ± 0.0003
Hydroximinogluconolactam (**3**)	1.28000 (± 0.064)



Reversible inhibitors

Some indications of positively charged transition states are also found in the inhibition studies performed. 1-Deoxynojirimycin and acarbose were shown to be potent inhibitors for GH family 31 enzymes (Hanozet et al., 1981; Chambers et al., 1982). Likewise, these compounds turned out to be potent competitive inhibitors for GLase with K_i values of 0.13 (1-deoxynojirimycin) and 0.020 (acarbose) μM, respectively (Table 2) (Lee et al., 2003). The potent, competitive inhibition by 1-deoxynojirimycin suggests that a cationic species has high affinity for the active site of GLase while the highly potent nanomolar inhibition exhibited by acarbose (K_i = 20 nM), itself a proven transition state analogue in-

Table 3. Kinetic isotope effects (KIE) measured for deuterated substrates with *Gracilariopsis* α-1,4-glucan lyase (Lee et al., 2002; 2003).

Substrate	KIE upon k_{cat}	KIE upon k_{cat}/K_m
1-[^{2}H]-PNPαGlc	1.19 ± 0.02	
1-[^{2}H]-αGlcF	1.14 ± 0.05	1.16 ± 0.01
1-[^{2}H]-5FαGlcF	1.23 ± 0.10	
2-[^{2}H]-PNPαGlc	1.06 ± 0.01	
2-[^{2}H]-αGlcF	1.06 ± 0.04	1.07 ± 0.01
2-[^{2}H]-5FαGlcF	1.92 ± 0.15	

hibitor (Mosi et al., 1998), strongly supports reaction via an oxocarbenium ion-like transition state. Hydroximinogluconolactam has been shown previously to be a potent inhibitor of GH family 13 α-glucosidase (K_i = 2.9 μM with yeast α-glucosidase) (Hoos et al., 1997). In the case of GLase, this compound was shown to be a poor inhibitor (K_i = 1.28 ± 0.06 mM). This poor binding affinity of hydroximinogluconolactam was also observed for α-glucosidase from *A. niger* of GH family 31 (K_i = 1.7 mM) (unpublished results) but remains unexplained.

Kinetic isotope effects

Further evidence for this mechanism is derived from kinetic isotope effect (KIE) analysis. Kinetic isotope effects have proved to be some of the best probes of transition state structure for mechanistic studies of retaining glycosidases (Sinnott, 1990; Zechel & Withers, 2000). By introducing deuterium either at C1 or C2, KIE analysis can directly reveal the structure of the transition state. The KIE measured for deuterium substitution on C-1 is an α-secondary deuterium KIE, which reports on the existence and extent of oxocarbenium ion-like character in the transition states of either the first or second step. The KIE measured for the 2-deutero substrates, on the other hand, would reveal the features of the transition state of the elimination reaction. Depending on whether or not the rate-limiting step involves proton transfer from C2, either a primary KIE or a β-secondary KIE would be expected from the 2-position. Combined with the KIE from the 1-position, the KIE from 2-position could provide insight into the transition state for the elimination step as has been done in the study of non-enzymatic elimination reactions (Fry, 1972).

Kinetic isotope effects measured for *p*-nitrophenyl α-D-glucopyranoside, α-D-glucopyranosyl fluoride and 5-fluoro-α-D-glucopyranosyl fluoride, substituted with deuterium, separately, at the 1- and 2-positions were reported (Table 3) (Lee et al., 2002; 2003). The first, glycosylation, step is rate-limiting for PNPαGlc as shown by the Brønsted relationship and the second, elimination, step is rate-limiting for 5FαGlcF while the rate-limiting step is unknown for αGlcF. Values of KIEs upon k_{cat} at C1 and C2 are of the expected magnitude for PNPαGlc, for which the first step (glycosylation) is rate-limiting. The value of KIE upon k_{cat} measured for 1-[^{2}H]-PNPαGlc corresponds to an α-secondary KIE and the large value measured indicates that there is a large degree of oxocarbenium ion character at the transition state. Similarly large α-secondary KIEs (1.12-1.21) have been reported previously for the hydrolysis of *p*-chlorophenyl α-glucopyranoside by sucrase-isomaltase and isomaltose by α-glucosidases from sugar beet and *A. niger*, all belonging to GH family 31 (Cogoli & Semenza, 1975; Igaki & Chiba, 1989), indicating that closely related mechanisms are followed between two different types of enzyme in the same gene family. More diagnostically useful are the KIE values measured for the 2-deutero substrate. If GLases adopted a directly operating *anti*-elimination reaction mechanism, a significant primary kinetic isotope effect would be expected. However, the observed value with 2-[^{2}H]-PNPαGlc (1.06) is not big enough for a primary KIE and presumably represents a β-secondary KIE. This is fully consistent with the trapping of the covalent intermediate. The similarity of the KIEs measured upon k_{cat} for PNPαGlc and k_{cat}/K_m for αGlcF implies that the same step is rate-limiting in the two cases. Since k_{cat}/K_m monitors the first irreversible step, this finding is fully consistent with the observation from the Brønsted relationship that the formation of the glycosyl-enzyme is rate-limiting for PNPαGlc. Interestingly, the equivalence of the KIE values on k_{cat} and k_{cat}/K_m for αGlcF implies that the same step is rate-limiting for both, and, since k_{cat}/K_m reflects the glycosylation step, thus implies that despite the k_{cat} value of 505 s^{-1}, glycosylation is still rate-limiting for k_{cat} with this substrate.

In summary, in all three cases where the first step, the formation of the covalent glycosyl-enzyme intermediate, is rate-limiting, large α-secondary KIEs have been measured from 1-deutero substrates, indicating very substantial oxocarbenium ion character at the transition state of the displacement reaction. These are fully consistent with those measured on other GH family 31 glycosidases. The absence of a primary KIE but the presence of a β-secondary KIE on 2-deutero substrates confirms that a direct *anti*-elimination mechanism is not in operation.

When all of the experimental results discussed earlier are combined with these KIE values, the following features for the transition state of the first step can be deduced: low β_{lg} observed in the Brønsted relationship of pK_a vs. log k_{cat} and log k_{cat}/K_m, large $\Delta\Delta G^{\ddagger}$ calculated from k_{cat}/K_m of fluorosugars, highly potent inhibition by transition state analogues, acarbose and 1-deoxynojirimycin, and large α-deuterium KIEs. Low β_{lg} values could mean either a small degree of glycosidic bond cleavage or early protonation. However, all other evidence points to a large degree of bond breaking at the transition state. In a modeling study on the

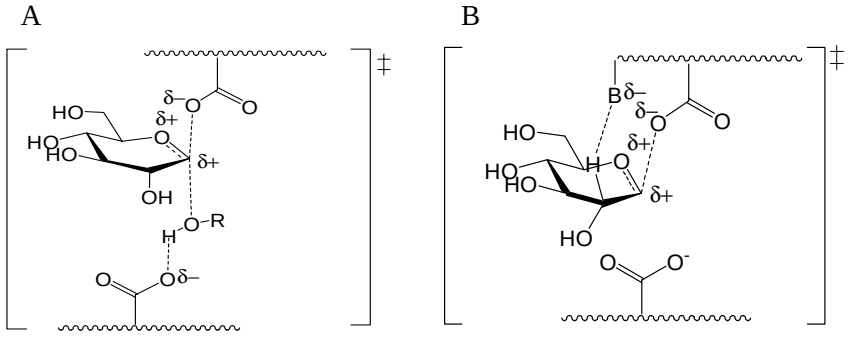


Fig. 12. Schematic diagram of the transition states. (A) The first step of the reaction catalyzed by GLase: the glycosidic bond is substantially broken to give a highly positively charged oxocarbenium ion-like transition state while the negative charge on the glycosidic oxygen is masked by proton donation. (B) The second step of the reaction (elimination) catalyzed by GLase: the glycosidic bond cleaves substantially to give a highly positively charged oxocarbenium ion character, making the β-hydrogen sufficiently acidic to be abstracted.

GH family 31 sugar beet α-glucosidase, 70% of the glycosidic bond was cleaved at the transition state for the hydrolysis of αGlcF and almost no bond formed from the incoming enzymatic nucleophile, indicating highly substantial oxocarbenium ion character (Tanaka et al., 1994). A similar transition state is predicted for GLase. Thus, it is likely that the glycosidic oxygen is protonated extensively, almost masking the developed negative charge, while the glycosidic bond between the anomeric center and the glycosidic oxygen is broken considerably to give substantial oxocarbenium ion character at the transition state of the first step in the degradation of α-1,4-glucans by GLase (Fig. 12A).

In contrast to the above KIE results, KIEs upon k_{cat} at each center measured with 5FαGlcF are quite different since the second step is suggested to be rate-limiting for 5FαGlcF unlike PNPαGlc and GlcF (Table 3) (Lee et al., 2003). A very large α-deuterium KIE of $k_H/k_D = 1.23$ was reported for 1-[^{2}H]-5FαGlcF, consistent with fully developed oxocarbenium ion character at the transition state. This is accompanied by a substantial isotope effect of $k_H/k_D = 1.92$ for the 2-deutero substrate (2-[^{2}H]-5FαGlcF). This value is clearly outside the range of a β-secondary isotope effect, and within the range of a primary kinetic isotope effect, thereby providing the first direct kinetic proof of a two-step mechanism for which the second step involves at least partially rate-limiting C-H bond cleavage. This primary KIE is relatively small for a fully concerted, synchronous E2 mechanism while the KIE measured with the 1-deutero derivative is quite large for an α-secondary deuterium KIE. Based on this pair of values, some distinction was made between stepwise and concerted mechanisms for the elimination step. The presence of a primary KIE at C2 eliminates the possibility of a fully stepwise E1 mechanism anticipated by the large secondary α-KIE. An E1 mechanism involving anchimeric assistance by the β-hydrogen and nucleophilic attack of solvent on the β-hydrogen has been previously shown to yield small primary KIE values of $1.72 \sim 1.85$ (Cram & Tadanier, 1959). However, such a mechanism is not possible in this case since the β-hydrogen is located on the same face of the pyranose ring as the scissile bond. Thus, a concerted mechanism with an asymmetric transition state involving extensive cleavage of the glycosidic bond and substantial proton transfer is suggested as a more probable mechanism and consistent with oxocarbenium ion chemistry. A small primary KIE might mean that the proton is either less than half or more than half transferred at the transition state. The latter case is inconsistent with substantial positive charge at the anomeric center, thus delayed proton transfer seems more probable. Indeed a mechanism in which substantial heterolysis of the glycosidic bond occurs will generate a species with substantial oxocarbenium ion character, thereby acidifying the β-hydrogen to be transferred. (Fig. 12B). The E2 mechanism is not necessarily completely synchronous and there can be a broad spectrum from carbocation-like to completely synchronous to carbanion-like in the E2 mechanism (More O'Ferrall, 1970; Fry, 1972). Such an E2 mechanism leaning towards E1 was previously invoked to explain low KIE values ($2.4 \sim 2.6$) of some non enzymatic E2 reactions (Bunnet et al., 1962) and a similar kind of E2 enzymatic *syn*-elimination was found in UDP-N-acetylglucosamine 2-epimerase, where a primary KIE of 1.8 was measured (Morgan et al., 1997).

Speculations on the general base acting in the second step

The identity of the base responsible for H-2 abstraction is not clear at this stage, but the following possibility should be taken into consideration. A strong candidate for this role must be the departing carboxylate oxygen of the catalytic nucleophile itself (Asp553). This group is correctly positioned on the β-face of the sugar and, since the transition state is late, will be available to act as a base (Fig. 13). Indeed the recently determined three-dimensional structure of an intermediate trapped on a GH family 31 α-xylosidase reveals that the carbonyl oxygen of the nucleophile is indeed situated in close proximity to that same H-2, though in that case, of course, an elimination does not occur (Lovering et al., 2005). Additional support for this notion comes from the observation that retaining glycosidases catalyze the hydration of glycal substrates via a covalent glycosyl-enzyme intermediate and do so via the *syn*-addition of the proton at C-2 and the enzymatic nucleophile. It has been generally assumed that the nucleophile itself donates this proton in a some-

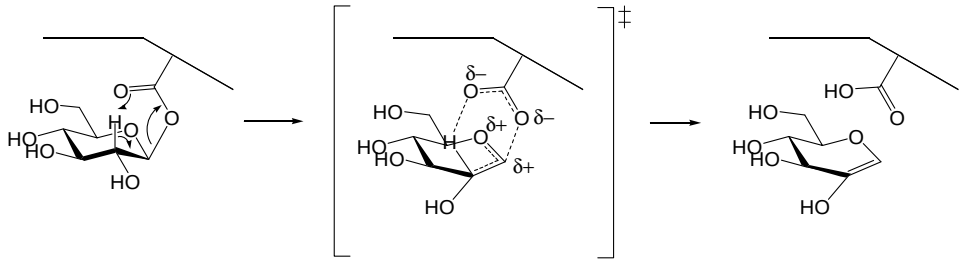


Fig. 13. Schematic diagram of a possible proton abstraction mechanism involving the catalytic residue which acts as a catalytic nucleophile in the first step of the reaction catalyzed by GLase.

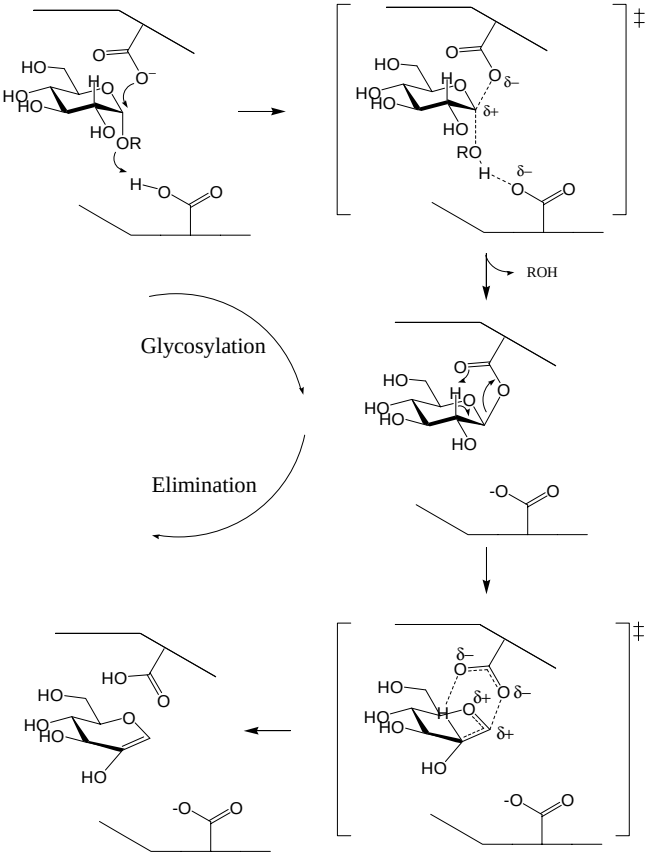


Fig. 14. Proposed mechanism of α-1,4-glucan lyase.

what concerted process highly analogous to that proposed for GLase (Legler, 1990). Wolfenden's observation that the ribosylation reaction catalyzed by nucleoside 2-deoxyribosyltransferase is accompanied by an occasional elimination reaction, generating D-ribal (Smar et al., 1991) also points to the existence of a mechanistic continuum.

Conclusion

The mechanism of GLase is suggested to be as depicted in Figure 14, highly similar in many aspects to that of α-glucosidases of GH family 31. Key features include the formation and base-catalyzed elimination of a covalent glycosyl-enzyme intermediate and highly positively charged transition states for both reactions. GLase utilizes mechanistic machinery that is common to GH family 31 and represents an example of mechanistic plasticity by which enzymatic mechanisms can evolve through subtle changes in active site constitution. The studies on GLase highlight the advantages of the sequence-based classification of glycosidases in considering reaction mechanisms and provide further caution about the annotation of genomes purely on the basis of sequence similarity. This novel mechanism for glycosidic bond cleavage showcases one of the unusual mechanisms adopted by nature to degrade sugars. Others include polysaccharide lyases and GH family 4 (Rye & Withers, 2002; Yip et al., 2004; Yip & Withers, 2004). These examples illustrate the diversity of approaches adopted for a common task.

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