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CONCISE ARTICLE

A Mechanistic Study on the α-*N*-acetylgalactosaminidase from *E. meningosepticum*: A Family 109 Glycoside Hydrolase

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A recombinant glycoside hydrolase family 109 α-*N*-acetylgalactosaminidase from the pathogenic bacteria *E. meningosepticum* catalyses the hydrolysis of aryl 2-acetamido-2-deoxy-α-D-galactopyranosides. The sensitivities to leaving group abilities (β_{lg} values) on *V* and *V*/*K* are –0.08 ± 0.06 and –0.31 ± 0.12, respectively. These results are consistent with an E2 elimination following hydride transfer from C3.

Introduction

There is a growing awareness in the scientific community that post-translational protein glycosylation is a complex modification, found throughout nature,[1] that individualizes biomolecules and imparts physical properties that define biomolecular roles in a range of physiological and biological recognition events. α-*N*-Acetylgalactosamine (αGalNAc) residues are commonly found as constituents of mucin glycoproteins,[2, 3] and they are critical antigenic components of blood group A[4] and of the sensory nerve structures of humans.[5] Enzymatic removal of the antigenic αGalNAc residue from blood group A cells has been studied as a potential route for the production of universal red blood cells (RBCs) for transfusion.[6-8] Glycosidases (glycoside hydrolases: GHs) constitute a superfamily of enzymes that hydrolyse glycosidic bonds found in a wide range of glycoconjugates. Based on sequence alignment, these enzymes are classified into more than 130 different families.[9-11] α-*N*-Acetylgalactosaminidases (α-NAGALs) are carbohydrate-processing enzymes that cleave terminal non-reducing αGalNAc residues from glycoconjugates. α-NAGALs have been classified into GH families 27, 36, 109 and 129.[8, 12-14] Of these, GH27 and GH36 share a common ancestral gene and are members of the glycoside hydrolase clan GH-D, which possess a commonly found triosephosphate isomerase (TIM) barrel structure.[9]

Most GHs[15] catalyze simple nucleophilic substitution reactions that occur on the anomeric carbon atom of a carbohydrate. The enzymatic transition state for this reaction involves the development of positive charge on the anomeric carbon, which then can delocalize onto the ring oxygen atom.[16, 17] Indeed, three out of the four glycoside hydrolase families that have α-NAGAL enzymes as members (GH27, GH36 and GH129) are proposed to operate via such nucleophilic substitution reactions.[12, 14]

In 2007, Liu *et al.* reported the X-ray crystal structure of an α-NAGAL enzyme from *E. meningosepticum,* which is a member of the exclusively prokaryotic GH109 family.[8] The X-ray diffraction structure revealed several similarities between GH4 glycosidases and GH109 α-NAGALs, as well as a remote relationship to the sequences of oxidoreductases.[8, 18] An unusual feature of the GH4 family is that various cofactors are required for activity, in particular, NAD^+, a divalent metal ion (Mn^{2+}) and a reducing agent (such as DTT or TCEP).[19-22] Scheme 1 shows the currently accepted mechanism for GH4 NAD^+-dependent glycosidases, using α-galactosidase as an example; the NAD^+-dependent glycosidase reaction mechanism is atypical of glycosidases. In Scheme 1, the enzyme:ketone complex is shown as a bona fide intermediate,[20-22] however, based on kinetic isotope effect evidence it has been proposed that the E:S complex undergoes a concerted reaction to give the E:enediolate intermediate directly.[19] Key observations that underpin this mechanism include: (i) a requirement for NAD^+ for activity, (ii) the first formed product has the same anomeric configuration as the substrate (retaining glycoside hydrolase), and (iii) when the reaction is performed in D_2O the hydrolysis product contains a deuterium on C-2.[20-22] Given that Liu et al. reported similar observations for their *E. meningosepticum* GH109 α-NAGAL it is clear that in addition to sharing a structural similarity, GH4 and GH109 enzymes are mechanistically comparable.[8, 23] Lastly, the GH4 family contains enzymes that catalyze the hydrolysis of both α- and β-glycoside substrates,[18, 24] an unusual situation that is not seen in GH109 where all members characterized to date are α-NAGALs that show strong substrate selectivity.[8]

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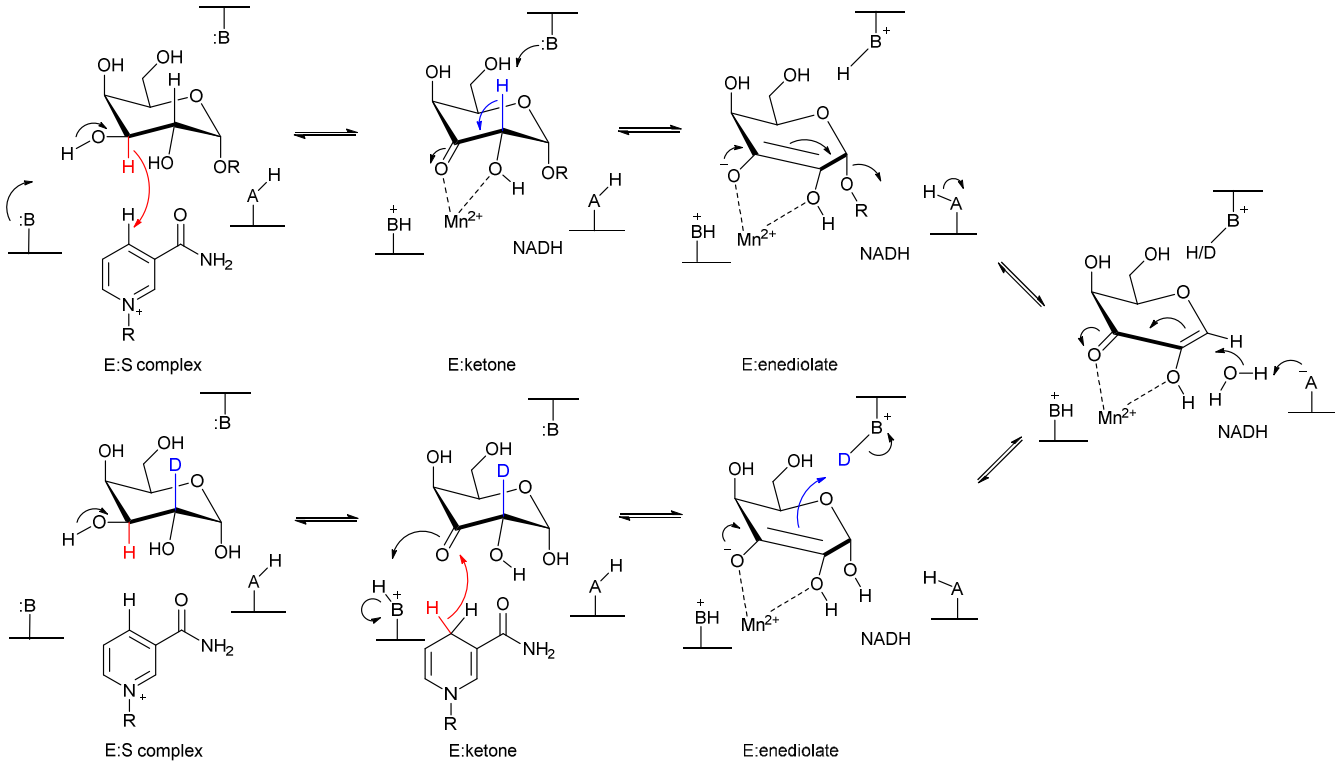


Scheme 1 Proposed mechanism for GH4 glycoside hydrolases showing all possible intermediates in the catalytic cycle. Hydride transfers are shown in red, while proton transfers to and from C2 are shown in blue.[19-22] The required divalent cation (Mn^{2+}) is not shown in all structures in order to avoid overlap with mechanistic arrows. Also shown is the incorporation of deuterium at C2 in the product when the reaction is performed in D_2O.

In contrast to GH4 glycosidases, the GH109 family enzyme *E. meningosepticum* α-*N*-acetylgalactosaminidase requires no external cofactors for activity. That is, the required NAD^+ is embedded in the enzyme environment where it remains tightly bound to the protein.[8] Because the *E. meningosepticum* enzyme does not require a divalent cation, the pK_a of the C2 proton of the intermediate (E:ketone, Scheme 1) is likely to be higher than that of the corresponding species bound to the GH4 enzyme. In contrast, the electrophilic stabilization provided by the bound divalent cation (Mn^{2+}) in GH4 enzymes should provide a dramatic lowering of the free energy of the E:enediolate intermediate (Scheme 1). This major difference in the GH4 and GH109 enzyme active sites piqued our interest and provided incentive for undertaking an in-depth mechanistic study on a GH109 family member. In this report, we detail the cloning of the Flv109 gene from *E. meningosepticum* (ATCC 51720D) as well as the expression, purification, and characterization of the encoded protein (α-NAGAL).

Enzyme Production

We amplified the genomic DNA of *E. meningosepticum*—using a different strain than that reported previously in the literature[8]—and used this material to generate a truncated α-*N*-acetylgalactosaminidase lacking the first 17 *N*-terminal amino acid residues. In addition, our construct differs in sequence from the enzyme used by Liu et al[8] by six amino acid residues. Full experimental details and protein and DNA sequences are given in the supporting information section. We mapped the six amino acid changes onto the published structure of the *E. meningosepticum* α-*N*-acetylgalactosaminidase (Fig. 1) and found that none are within the active site of the enzyme.

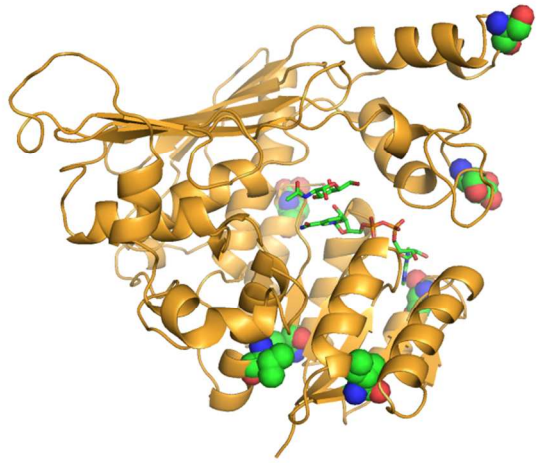
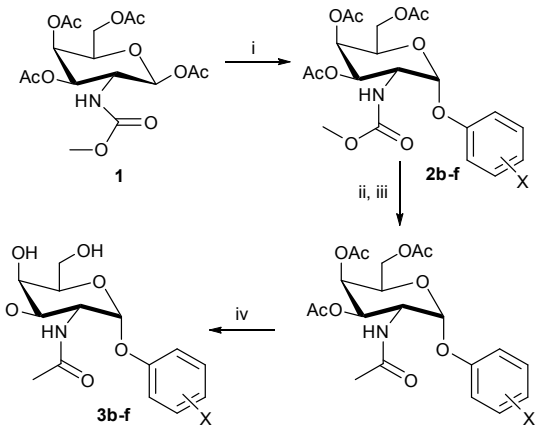


Fig. 1 The structure for a single monomer of the *E. meningosepticum* α-*N*-acetylgalactosaminidase, where the NAD^+ and GalNAc bound to the active site are shown as stick structures. The positions of the six amino acid residues that differ between the α-NAGAL used in the current study and that reported by Liu et al. are shown using space-filling structures.

Substrate Synthesis.

A panel of five substituted aryl 2-acetamido-2-deoxy-α-D-galactopyranosides were synthesized and used in combination with the commercially available 4-nitrophenyl substrate (**3a**) to assess the effect of leaving group ability on the two catalytic constants (k_{cat} and k_{cat}/K_m). We used stannic chloride promoted glycosylation of methylcarbamate **1** for the synthesis of the protected aryl α-galactosaminides **2b–f** (Scheme 2). Standard reactions gave access to substrates **3b–f**. Full experimental details are given in the supporting information section.



Scheme 2 Reagents and conditions for the syntheses of aryl 2-acetamido-2-deoxy-α-D-galactopyranoside substrates: (i) ArOH, $SnCl_4$, CH_2Cl_2, rt; (ii) TBAF, THF, reflux; (iii) Ac_2O, pyridine, rt; (vi) NaOMe, MeOH, 0 °C.

Kinetic and Product Studies.

In order to verify mechanistic commonality between the enzymes of glycoside hydrolase families 4 and 109 (GH4 and GH109) and to confirm that the six amino acid changes to our enzyme are mechanistically unimportant we performed a product study for the α-NAGAL-catalyzed hydrolysis of 4-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (PNPαGalNAc) in the presence of methanol. The product of this reaction contained an anomeric proton with a chemical shift of 5.22 ($J_{1,2}$ = 3.8 Hz), thereby confirming that the reaction proceeded with retention of anomeric configuration to give methyl 2-acetamido-2-deoxy-α-D-galactopyranoside. We also confirmed that α-NAGAL-catalyzed hydrolysis of PNPαGalNAc in D_2O gave the reaction product 2-acetamido-2-deoxy-D-galactose which was completely deuterated at C-2 as indicated by the appearance of the anomeric protons for both anomers as singlets in the ^1H NMR spectrum.

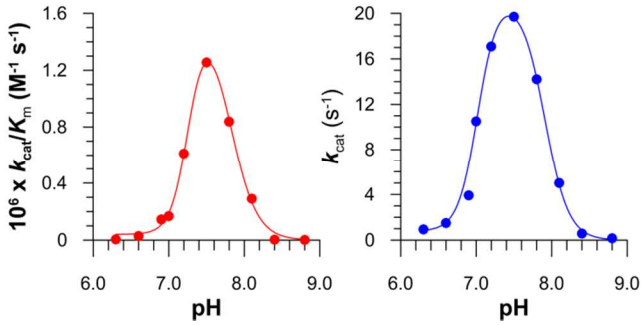


Fig. 2 pH activity profiles: k_{cat}/K_m (left in red) and k_{cat} (right in blue) for the hydrolysis of PNPαGalNAc by α-NAGAL.

The measured pH versus rate profiles for k_{cat}/K_m and k_{cat} (**Fig. 2**) for the *E. meningosepticum* GH109 α-NAGAL-catalyzed hydrolysis of PNPαGalNAc are bell-shaped curves. In theory, a bell-shaped curve for the pH profile of k_{cat} means that the enzyme active site has two ionisable groups (one protonated and the other deprotonated during the catalytic cycle).[25] In the case of our enzyme, which exists in solution as a functional dimer, the experimental data only fit to a pH-rate equation that incorporated Hill coefficients on both of the ionization events (Table 1). One possibility for the derived Hill coefficients on the changes in α-NAGAL activity as a function of pH being greater than 2 is that the two active sites in the dimeric enzyme—one from each monomeric unit—are not functionally independent of one another.[25] Our GH109 α-NAGAL enzyme exhibits a narrow peak of activity in a pH range of 7.0–8.0 (Fig. 2), and the two apparent pK_a values that characterize its activity are around 7.1 and 7.9 (Table 1).

The maximal rate constants measured in our current study are similar to those reported by Liu et al. (Table 1).[8] Thus, we conclude that the six amino acid differences between the two enzymes do not significantly affect their catalytic activities.

Table 1 Kinetic Parameters for GH109 *E. meningosepticum* α-*N*-acetylgalactosaminidase-catalyzed hydrolysis of PNPαGalNAc.

parameter	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
maximal rate constants	19.7 ± 1.0^a	$(1.25 \pm 0.05) \times 10^{6a}$
pK_{a1}	7.0 ± 0.1	7.3 ± 0.1
pK_{a2}	7.9 ± 0.1	7.8 ± 0.1
h_1	3.4 ± 0.2	3.7 ± 1.4
h_2	2.8 ± 0.2	2.5 ± 0.8

[a] Rate constants reported by Liu et al. $k_{cat} = 9.84 \pm 0.16$ s^{-1} $k_{cat}/K_m = (1.28 \pm 0.10) \times 10^6$ M^{-1} s^{-1}.[8]

Next, we evaluated the sensitivity of the enzymatic rate constants k_{cat} and k_{cat}/K_m to a change in the leaving group ability. This was accomplished by measuring the change in the logarithm of the rate constant as a function of the pK_a of the leaving group aglycone's conjugate acid.[26] Fig. 3 presents Brønsted plots of the

data; the derived sensitivities (β_{lg} values) on k_{cat} and k_{cat}/K_m are –0.08 ± 0.06 and –0.31 ± 0.12, respectively.

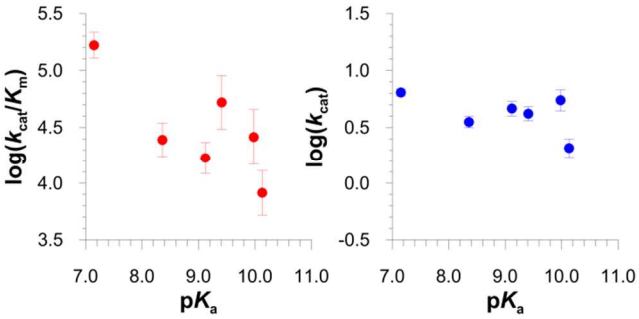


Fig. 3 Brønsted plots. Effect of leaving group ability on k_{cat}/K_m (red circles) and k_{cat} (blue circles) for α-*N*-acetylgalactosaminidase-catalyzed hydrolyses. All experiments were performed at 37 °C and pH 7.5. Leaving group abilities represented as pK_a (ArOH) are as follows: 4-nitrophenol (7.15); 3-nitrophenol (8.36); 3-chlorophenol (9.12); 4-chlorophenol (9.41); phenol (9.98); and 4-methoxyphenol (10.13).

The mechanism of action of GH4 and GH109 enzymes involves a hydride transfer from C3 to a bound NAD^+ co-factor to give a non-covalently bound ketone (Scheme 1). Following the formation of this intermediate,[20, 22] or during hydride transfer,[19] a proton is transferred from C2 of the sugar to an enzymatic tyrosine residue.[8, 18, 24] This transfer occur either simultaneously with (i.e., an E2 elimination mechanism) or prior to (i.e., an elimination reaction that proceeds via a conjugate base, $E1_{CBirr}$) aglycone departure to give a bound ketone-glycal intermediate (Scheme 1, E:glycal-ketone). The measured β_{lg} value on k_{cat}/K_m (–0.31 ± 0.12), is consistent with cleavage of the anomeric C–O bond being kinetically significant, although scatter in the Brønsted plot makes it difficult to come to a more definitive conclusion. If glycosidic bond cleavage is partially rate-determining then breaking this bond is likely to be part of a concerted E2 elimination reaction which by virtue of the good leaving groups used in this study, would occur without acid-catalysis. Mechanistic studies on GH4 enzymes report smaller β_{lg} values[20, 22] than our values for the GH109 enzyme; the GH4 results are interpreted to mean that the elimination occurs via an $E1_{CBirr}$ ($D_HAxh^{‡}$ + D_N)[27] reaction. For the GH4 enzymes deprotonation to form the E:enediolate intermediate is partially rate limiting for k_{cat}/K_m (Scheme 1).

Keeping in mind that GH4 enzymes require a divalent cation for activity, we suggest that their active site environment is such that the C-2 proton of the substrate is made more acidic as the enediolate intermediate undergoes electrophilic stabilization by the metal cofactor. In contrast, the GH109 α-NAGAL active site promotes binding with the C2 substituent via hydrophobic and hydrogen-bonding interactions with methionine and tyrosine residues, respectively (Fig. 4). We also note that the structure of α-*N*-acetylgalactosaminidase does not have an appropriately placed acidic residue to assist in aglycone departure.[8]

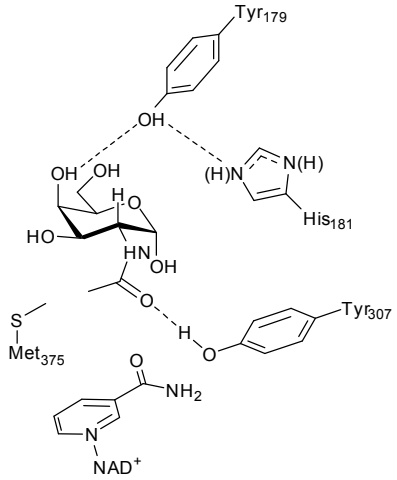


Fig. 4 Cartoon depiction of the active site of GH109 *E. meningosepticum* α-*N*-acetylgalactosaminidase (modified from references [8, 23]). Shown is the most likely base for the abstraction of the C2-proton (Tyr_{179}), presumably activated by the proximal His_{181}). Also, shown are the amino acids residues that interact with the C2-acetamido group: Tyr_{301} and Met_{375} via hydrogen-bonding and hydrophobic interactions, respectively.

Based on the β_{lg} value on k_{cat} of close to zero and if the Brønsted β_{lg} value on k_{cat}/K_m is non-zero, the kinetically significant step for k_{cat} occurs after glycosidic bond cleavage. This step possibly involves either the Michael addition of water to the glycal-ketone intermediate—which would likely occur through a network of solvent molecules due to the lack of an acid/base catalyst proximal to the glycosidic oxygen—or the subsequent transfer of either a proton or a hydride.

Given that both GH4 and GH109 enzymes are only produced by prokaryotes, the design of selective inhibitors for these two families of glycoside hydrolases is attractive from an antimicrobial therapeutics perspective.

Conclusions

The *E. meningosepticum* α-*N*-acetylgalactosaminidase hydrolyses 2-acetamido-2-deoxy-α-D-galactopyranosides by a NAD^+-mediated oxidation followed by an α,β-elimination to give a Michael acceptor intermediate. This intermediate undergoes hydration along with proton and hydride transfer to generate 2-acetamido-2-deoxy-α-D-galactose as the reaction product. The hydration reaction limits k_{cat}, and the E2 elimination is at least partially rate-limiting for k_{cat}/K_m. More detailed mechanistic conclusions concerning the hydride transfer to NAD^+ from C3 will require the development of new synthetic procedures to make C1, C2 and C3 deuterated substrates.

Notes and references

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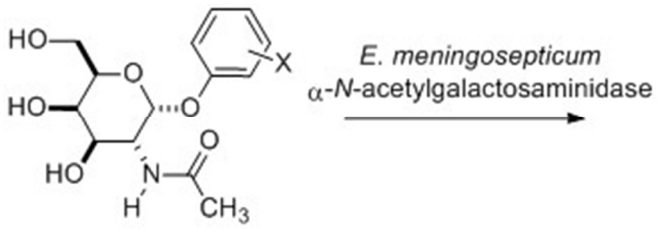

Studies on the mechanism of action used by a GH109 enzyme
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