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O-GlcNAcase Catalyzes Cleavage of Thioglycosides without General Acid Catalysis

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Thioglycosides are widely perceived as being impervious to glycosidase-catalyzed hydrolysis.[1] Accordingly, thioglycosides have gained widespread use as inhibitors of these enzymes,[2] ligands for affinity chromatography,[3] and have also been suggested for use as therapeutics.[4] The stability of thioglycosides to nearly all glycosidases is so marked that a number of co-crystal structures in which the thioglycosidic linkage is bound *intact* across the active site have been obtained.[5] The inability of most glycosidases to catalyze hydrolysis of thioglycosides[6] has been postulated to stem, in part, from the poor hydrogen bonding ability of the sulfur atom, which renders *S*,*O*-acetals unable to benefit significantly from general acid catalysis.[7a−c]

In Nature, thioglycosidase activity has been identified in extracts from several organisms.[8] Nevertheless, the only glycosidases with well-defined thioglycosidase activity having been identified are the plant myrosinases.[9] Elegant studies have shown that, unlike all other glycosidases which have a highly conserved general acid catalytic residue to facilitate departure of an alkoxide leaving group,[10] the family 1 myrosinases lack such a group.[11] Accordingly, myrosinases cleave activated *S*-glycosides bearing an *O*-sulfated thiohydroxamic acid aglycone for which a general acid cannot be expected to assist in catalysis.

Recently, we have shown that family 84 human *O*-GlcNAcase uses a catalytic mechanism involving anchimeric assistance (Figure 1).[12] This enzyme cleaves GlcNAc from post-translationally modified intracellular proteins.[13] In light of the apparent stability of thioglycosides to glycosidases,[6] we were surprised to observe that human *O*-GlcNAcase cleaved pNP-*S*-GlcNAc with comparable efficiency as pNP-*O*-GlcNAc.[14] Indeed, the ratio of the second-order rate constants $[(V_{max}/[E]_oK_M)_O/(V_{max}/[E]_oK_M)_S]$ for the *O*-GlcNAcase-catalyzed hydrolysis of these substrates is only ≈20 (Figure 2A). To address whether this large enzyme might contain a second active site, we used NAG-thiazoline, a competitive inhibitor of the *O*-glycoside activity of *O*-GlcNAcase,[12] and found identical K_I values for the cleavage of both pNP-*S*-GlcNAc and pNP-*O*-GlcNAc. Furthermore, we assayed the *O*-GlcNAcase-catalyzed hydrolysis of 4-methyl*thio*umbelliferyl 2-deoxy-2-*N*-fluoroacetyl-β-D-glucoside (Figure 2B). The steep negative slope in the Taft-like linear free energy analysis shows that the 2-acetamido group of the substrate participates in catalysis to a greater extent for the *S*-glycosides than found earlier for the corresponding *O*-glycosides.[12] Together, these results reveal that *O*-GlcNAcase uses the same active site and general catalytic mechanism to cleave both *S*- and *O*-glycosides.

Studies on the spontaneous hydrolysis of benzaldehyde *O*-ethyl *S*-phenyl acetal have previously shown that cleavage of the C−S bond in *S*,*O*-acetals does not benefit significantly from general acid catalysis, while the corresponding *O*-acetals do.[7] To evaluate the extent of acid catalysis provided by *O*-GlcNAcase for *S*- and *O*-glycosides, we assayed two series of substrates bearing different substituted thiophenol or phenol leaving groups. The resulting Brønsted analyses provide insight into the development of effective charge at the exocyclic heteroatom in the transition state. For both series, strong linear relationships between the second-order rate

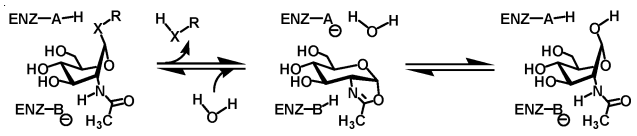

Figure 1. *O*-GlcNAcase uses a catalytic mechanism involving substrate-assisted catalysis from the 2-acetamido group of the substrate to form a transient oxazoline intermediate. Departure of oxygen leaving groups (X = O) is aided by a general acid catalytic residue within the enzyme. For leaving groups bearing sulfur (X = S), there is no acid catalysis.

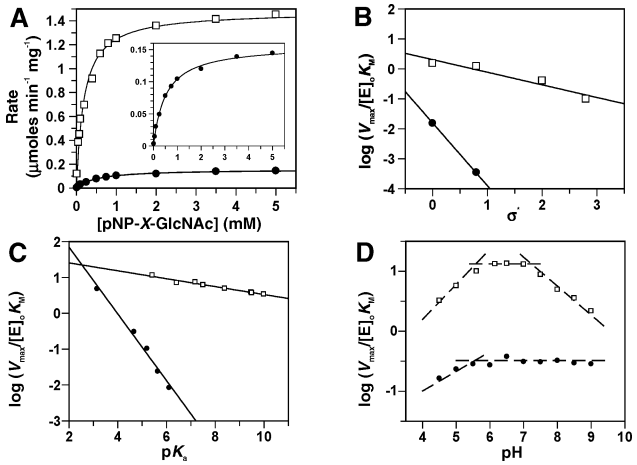

Figure 2. *O*-GlcNAcase uses substrate-assisted catalysis to effect *S*- and *O*-glycoside cleavage. (A) Initial velocities from the cleavage of pNP-*S*-GlcNAc, X = S (●), and pNP-*O*-GlcNAc, X = O (□). Inset: Detail of the region showing cleavage of the pNP-*S*-GlcNAc. (B) Taft-like analyses of $(V_{max}/[E]_oK_M)$ versus the Taft parameter (σ^*) for *O*-GlcNAcase-catalyzed hydrolysis of pNP-*O*-GlcNAc (□, data taken from ref 12) or pNP-*S*-GlcNAc (●) substrates bearing different *N*-fluoroacetyl groups. *O*-GlcNAcase does not use acid catalysis to facilitate cleavage of *S*-glycosides but does for *O*-glycosides. (C) Brønsted plots for *O*-linked (□) and *S*-linked (●) substrates. (B) pH dependence for catalysis of pNP-*O*-GlcNAc (□) and pNP-*S*-GlcNAc (●).

constant for each substrate and the pK_a value of its corresponding leaving group are observed (Figure 2C). The shallow slope (β_{lg}-(V/*K*) = −0.11 ± 0.01) observed with the *O*-glycosides indicates little negative charge accumulation on the exocyclic oxygen and suggests two possible mechanistic scenarios. The first is a late transition state wherein both cleavage of the glycosidic bond and proton donation are significantly advanced. The second possibility is an early transition state, with both little C−O bond cleavage and little proton donation. The first scenario is most likely since it is consistent with the general structures of transition states for the glycosidase superfamily,[10] including family 20 hexosaminidases,[15] and agrees with the large $^{\alpha-D}$(V)-KIE values we describe below. In marked contrast, for the series of *S*-glycosides a steep slope is seen (β_{lg}(V/*K*) = −0.93 ± 0.06), indicating pronounced negative charge accumulation on the exocyclic sulfur atom. Such a steep negative slope suggests that the *O*-GlcNAcase-catalyzed hydrolysis of thioglycosides involves little, if any, general acid catalysis, and that the reaction proceeds through a transition state in which the

leaving group has essentially dissociated from the anomeric center. Interestingly, such large negative $\beta_{lg}(V/K)$ values (≈ -1) have been observed for wild-type retaining glycosidases as well as for mutants in which the acid/base carboxyl group has been deleted.[10]

Two further sets of data support the view that no acid catalysis is used by *O*-GlcNAcase for cleavage of *S*-glycosides. The first set is the pH profiles for the cleavage of pNP-*S*-GlcNAc and pNP-*O*-GlcNAc (Figure 2D). The bell-shaped curve for cleavage of pNP-*O*-GlcNAc is characteristic of the very large majority of glycosidases and arises from the ionization of two key catalytic residues within the active site of *O*-GlcNAcase (Figure 1).[10] The pH profile for pNP-*S*-GlcNAc, on the other hand, reveals that only one ionization is important for catalysis. Such an asymmetric pH profile resembles those of some glycosidases in which the acid/base residue has been mutated to a nonionizable group.[10] Second, a mutant of *O*-GlcNAcase lacking the general acid/base residue catalyzes the hydrolysis of *S*- and *O*-glycosides at nearly identical rates.[16] Taken together, these data provide strong evidence that *O*-GlcNAcase uses acid catalysis to facilitate cleavage of *O*-glycosides but not *S*-glycosides, an observation entirely in accord with previous studies showing that nonenzymatic hydrolysis of *S*,*O*-acetals also does not benefit significantly from general acid catalysis.[7]

This lack of general acid catalysis toward sulfur is likely common for all glycosidases, yet this deficiency cannot, on its own, account for the apparent inability of most glycosidases to cleave *S*-glycosides. Because most glycosidases cannot cleave even highly activated thioglycosides,[6] these enzymes must generally be unable to effectively stabilize a transition state bearing a sulfur atom in the exocyclic position. This situation does not apply to *O*-GlcNAcase. Indeed, the $[(V_{max}/[E]_oK_M)_O/(V_{max}/[E]_oK_M)_S]$ ratio for the hydrolysis of phenyl-*O*-GlcNAc versus that calculated for phenyl-*S*-GlcNAc is only 900. This value is comparable with a ratio of 700 previously measured for the spontaneous hydrolysis of benzaldehyde *O*-ethyl-*O*-phenyl acetal versus that for the related *O*-ethyl *S*-phenyl acetal.[7c] Together, these results reveal that *O*-GlcNAcase is a proficient bifunctional catalyst, with likely similar $(k_{cat}/K_M)/k_{uncat}$ values for both *S*- and *O*-glycosides.

An interesting question is what makes *O*-GlcNAcase an efficient catalyst toward both *S*- and *O*-glycosides, with comparable rate accelerations, while most glycosidases appear unable to cleave *S*-glycosides at all.[1,6] Previously, large α-D KIE values have been observed for the pH-independent spontaneous hydrolysis of benzaldehyde *O*-ethyl *S*-phenyl thioacetals (1.13 ± 0.02) and for the acetic acid catalyzed hydrolysis of the corresponding *O*-acetal (1.17 ± 0.03), suggesting that both C−S and C−O bond cleavage is significantly advanced in these transition states.[7b,d] We therefore measured $^{\alpha-D}$(V)-KIE values for the *O*-GlcNAcase-catalyzed hydrolysis of (1-^{2}H)-pNP-*O*-GlcNAc and (1-^{2}H)-pNP-*S*-GlcNAc. The k_H/k_D ratio was a very large 1.14 ± 0.02, for the *O*-glycoside and 1.20 ± 0.02 for the *S*-glycoside. This latter value is the largest measured to date for the glycosylation step of any glycosidase.[10] These large KIE's reveal *very* dissociative transition states and suggest that the transition state for *S*-glycoside cleavage may be later than that for *O*-glycoside cleavage. Such a view is consistent with the increased participation of the 2-acetamido group, as shown in the Taft analyses. However, direct comparisons between these KIE's are difficult since they likely report on different steps of the mechanism. As well, realization of the true intrinsic KIE's may be obscured by binding isotope effects[17] or by contributions from external commitments. Regardless, these values are large, and so it appears that *O*-GlcNAcase has evolved to stabilize unusually late transition states suited to facilitating hydrolysis of *S*-glycosides *without* acid catalysis and *O*-glycosides *with* acid catalysis.

There have been reports identifying *S*-glycoside-modified proteins in mammals.[18] Although it remains to be determined if the *O*-GlcNAc transferase (OGTase) is capable of modifying a cysteine residue with GlcNAc, it cannot be ruled out, especially considering that several *O*-glycosyl transferases catalyze glycosyl transfer to thiol acceptors with only approximately 100-fold lower catalytic efficiency.[19] These observations open an interesting possibility. *O*-GlcNAcase may have evolved some catalytic efficiency toward *S*-glycosides as a means of slowly degrading potentially toxic *S*-GlcNAc-modified proteins or peptides that may arise from catalytic promiscuity of OGTase. Alternatively, this thioglycosidase activity could be serendipitous, and future studies may reveal similar activities in other glycosidase families.

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Supporting Information Available: Details of assays, preparation of substrates, and tables of kinetic parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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