Thioglycoside Hydrolysis

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Family 4 Glycosidases Carry Out Efficient Hydrolysis of Thioglycosides by an α,β-Elimination Mechanism**

*Vivian L. Y. Yip and Stephen G. Withers**

Considerable efforts have been extended towards the development of glycosidase inhibitors, both as stable substrate analogues for structural and mechanistic studies and for potential therapeutic and industrial applications.[1,2] Amongst those developed, thioglycosides, in which the glycosidic oxygen has been replaced by a sulfur atom, have proved to be stable analogues of the ground-state substrate and have been employed in a number of insightful structural studies.[3,4][*] Glycosidases are known to effect hydrolysis by acid/base-catalyzed mechanisms involving oxocarbenium-ion-like transition states.[5–7] Consequently, the resistance of the thioglycosidic bond to cleavage has been ascribed to the lower proton affinity of sulfur over that of oxygen, resulting in inefficient general acid catalysis to the departing aglycone.[8–10]

[*] V. L. Y. Yip, Prof. S. G. Withers
Department of Chemistry
University of British Columbia
2036 Main Mall, Vancouver, BC V6T 1Z1 (Canada)
Fax: (+1) 604-822-8869
E-mail: withers@chem.ubc.ca

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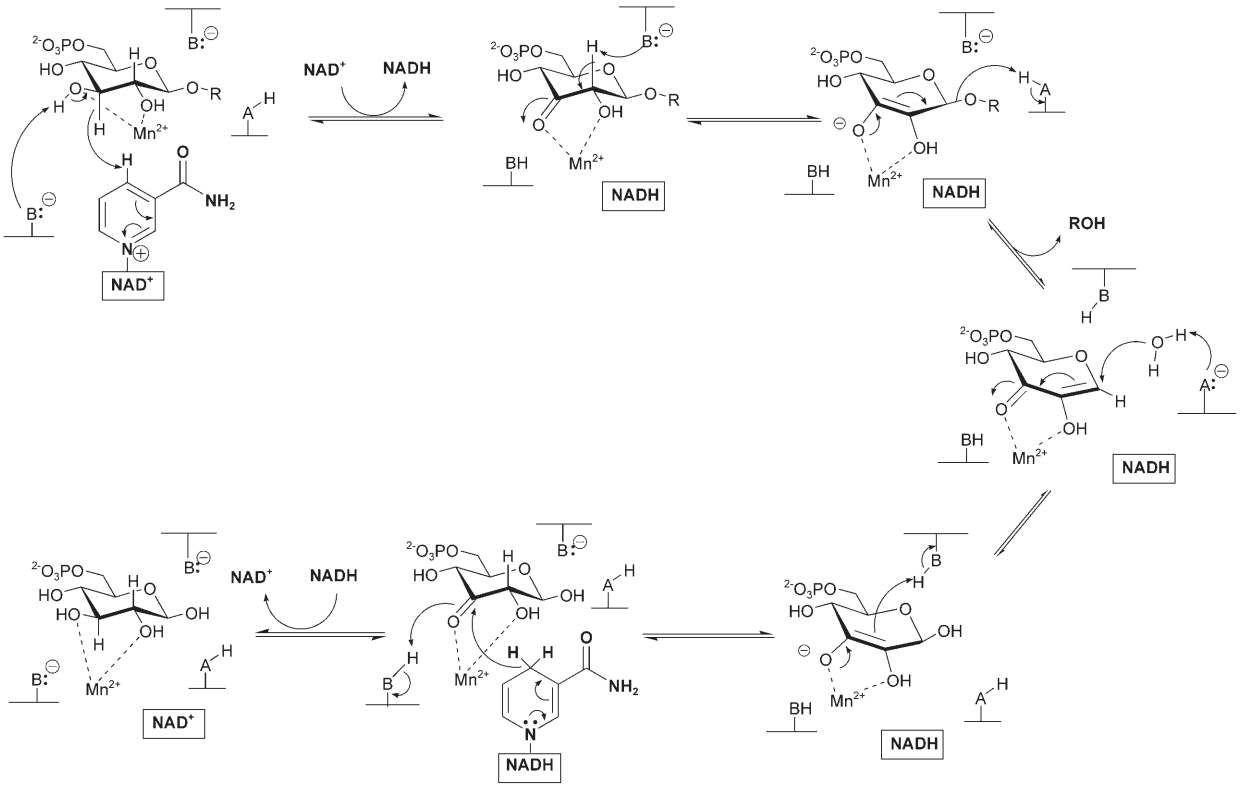
[*] The one exception is a specialized group of *S*-glycosidases of plant origin, called the myrosinases (E.C. 3.2.3.1), which specifically hydrolyze glucosinolate substrates, anionic 1-thio-β-glucosides. By sequence aligment, the myrosinases are associated with the family 1 glycosidases, which catalyze the hydrolysis of β-*O*-glycosides with retention of the substrate anomeric configuration. While the majority of family 1 glycosidases contain a conserved glutamate as the catalytic nucleophile, in the myrosinases the glutamic acid that serves as the acid/base catalyst is replaced by a glutamine residue. It has been proposed that the myrosinases are able to catalyze the cleavage of glucosinolate, because the substrates contain inherently good leaving groups and thus do not require general acid assistance for leaving-group departure.[25] A bound ascorbate anion appears to function as the general base catalyst for hydrolysis of the glycosyl–enzyme intermediate. A similar explanation is given for the GH84 human *O*-GlcNAcase, which is proposed to catalyze the hydrolysis of activated thioglycosides via a very dissociative transition state and without general acid catalysis to protonate the thiolate leaving group.[21]

The enzymes that have evolved to cleave carbon–sulfur linkages generally appear to use an anionic elimination mechanism. Excellent examples include cysteine Cβ-Sγ lyases,[11] β-cystathionase,[12] *S*-(β-aminoethyl)-cysteine,[13] *S*-alkylcysteine lyase,[14,15] *S*-adenosyl homocysteine hydrolase (AdoHCyase),[16] and the more recently uncovered ribosyl homocysteinase.[17] Most significantly, AdoHCyase[16] utilizes an NAD^+ cofactor to oxidize the ribose C3 hydroxy group of *S*-adenosyl homocysteine, thereby lowering the pK_a of the C4 proton and facilitating deprotonation and hence α,β-elimination of the thiol. The Michael acceptor then undergoes 1,4 nucleophilic attack by a water molecule, followed by reduction of the C3 ketone by the "on-board" NADH, yielding adenosine and homocysteine and returning the enzyme to its initial catalytic state.

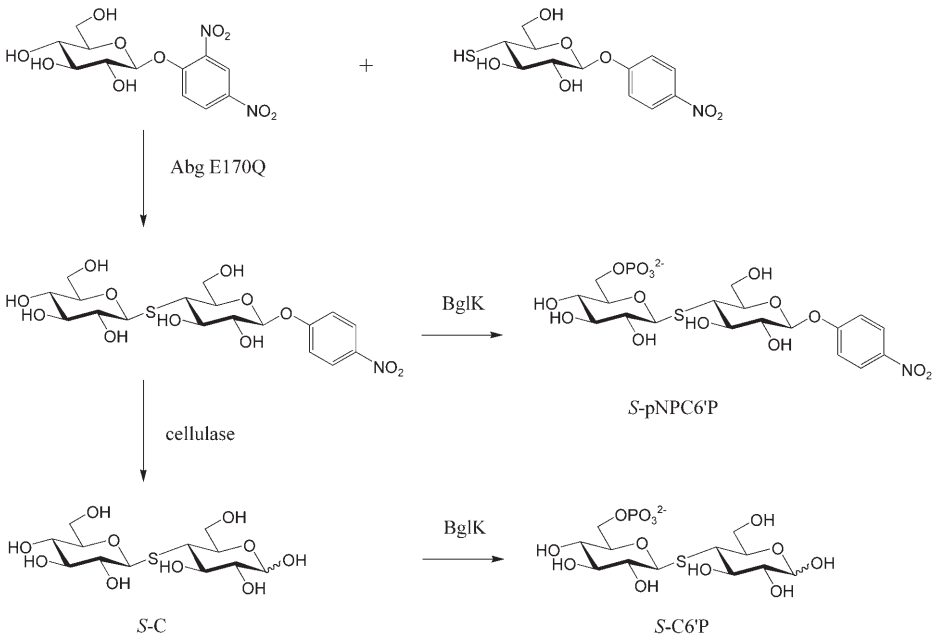
We recently unveiled a completely new mechanism of enzymatic glycoside hydrolysis analogous to that of AdoHCyase and also involving anionic transition states. The glycosidases that employ this mechanism are found within glycoside hydrolase family 4 (GH4), which also use an "on-board" NAD^+. In this case, the cofactor is used to transiently oxidize the C3 hydroxy group, as shown in Scheme 1.[18] Key features of the mechanism include oxidation of the C3 hydroxy group, formation of an enediolate intermediate stabilized by a bound Mn^{2+} cofactor, addition of water to the Michael acceptor so formed, and finally reduction of the ketone.

The similarities of the elimination mechanisms of AdoHCyase and glycosidases from family 4 raised the question as to whether GH4 enzymes, in contrast to all other glycosidases, can efficiently hydrolyze thioglycosidic bonds. Not only is this of fundamental mechanistic interest, but also it is highly relevant to the design of specific glycosidase inhibitors as potential therapeutics. Preliminary results[19] had indicated that thioglycosides might, in general, function as substrates since *p*-nitrophenyl 6-phospho-β-D-thioglucoside (*S*-pNPG6P) was shown to be hydrolyzed with kinetic parameters that are very similar to those of its oxygen-containing counterpart, *p*-nitrophenyl 6-phospho-β-D-glucoside (*O*-pNPG6P).[19] However, the use of an activated aryl leaving group could be misleading in this case as previous studies with a "normal" family 1 β-glucosidase, Abg,[20] and a GH84 human *O*-GlcNAcase[21] have shown that activated aryl thioglycosides could indeed be cleaved reasonably efficiently. To properly test whether family 4 enzymes can cleave thioglycosides, it was necessary to study the hydrolysis of non-activated thiodisaccharide substrates. Accordingly, we set out to synthesize and test thioglycoside analogues of the natural substrate for the *Thermotoga maritima* BglT, a 6-phospho-β-glucosidase from GH4 for which a three-dimensional structure and a number of mechanistic studies are available. To simplify assays, as well as synthetic routes, we elected to synthesize the *p*-nitrophenyl glycoside of thiocellobiose, as well as its oxygen-linked counterpart.

p-Nitrophenyl 4-deoxy-4-thio-6′-phospho-β-D-cellobioside (*S*-pNPC6′P) was synthesized according to the chemoenzymatic route shown in Scheme 2 starting with *p*-nitrophenyl 4-deoxy-4-thio-β-D-glucoside. The key step involved an enzymatic coupling using the thioglycoligase technology recently developed in our group to produce the thiodisac-



Scheme 1. Proposed mechanism of BglT, a 6-phospho-β-glucosidase from GH4.



Scheme 2. Synthesis of *S*-pNPC6′P and *S*-C6′P.

charide *p*-nitrophenyl 4-deoxy-4-thio-β-D-cellobioside (*S*-pNPC).[22] The free disaccharide, 4-deoxy-4-thio-D-cellobiose (*S*-C; Scheme 2), was prepared from *S*-pNPC by removal of the *p*-nitrophenyl group at C1 using the cellulase Onozuka R-10 from *Trichoderma viride*. The disaccharide products *S*-pNPC and *S*-C were then selectively phosphorylated at O6′ using the kinase BglK from *Klebsiella pneumoniae* that is associated with the phosphoenolpyruvate-dependent sugar: phosphotransferase system (PEP:PTS) used by many bacterial species for the simultaneous phosphorylation and translocation of carbohydrates into the cell.[23] The oxygen analogues, *p*-nitrophenyl 6′-phospho-β-D-cellobioside (*O*-pNPC6′P) and D-cellobiose 6′-phosphate (*O*-C6′P), were synthesized analogously from the *p*-nitrophenyl β-D-cellobioside and D-cellobiose, respectively, using the same kinase (Scheme 3).

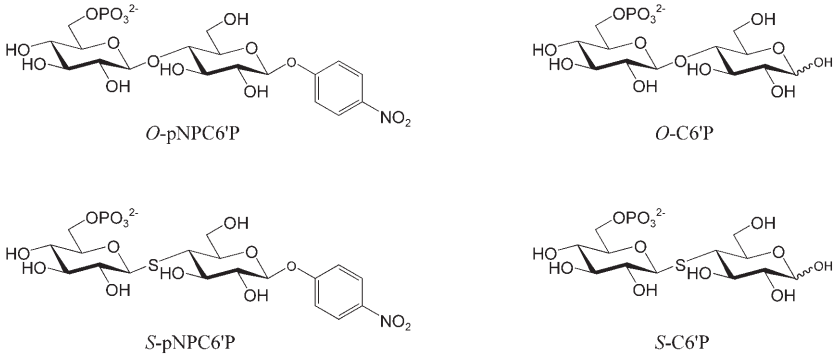
Kinetic parameters for all substrates were determined using a coupled assay system, in which the formation of the glucose 6-phosphate product was coupled to the reduction of NADP through inclusion of the enzyme glucose 6-phosphate dehydrogenase. A second, coupled assay system was also employed in which the *p*-nitrophenyl β-D-glucoside released from *O*-pNPC6′P was hydrolyzed by an added β-glucosidase. However, since *p*-nitrophenyl 4-deoxy-4-thio-β-D-glucoside is not a substrate for Abg, this coupled assay could not be used for *S*-pNPC6′P.

The kinetic parameters for the hydrolysis of the disaccharide substrates are shown in Table 1 along with K_i values for each disaccharide as an inhibitor of *O*-pNPG6P hydrolysis. The kinetic parameters for the disaccharide substrates are similar; the k_{cat} and K_M values for *S*-pNPC6′P are larger than those for *O*-pNPC6′P, resulting in similar k_{cat}/K_M values. Meanwhile, the k_{cat} and K_M values for *O*-C6′P are both slightly larger than those for *S*-C6′P, resulting in similar k_{cat}/K_M values. The similarities of the K_M and K_i values in each case confirms that the reaction is indeed occurring through the same active site. Furthermore, product analyses by NMR spectroscopy and mass spectrometry showed that the expected products (glucose 6-phosphate and 4-deoxy-4-thioglucose) are indeed formed when *S*-C6′P is reacted with BglT. In addition, deuterium incorporation from the solvent into the glucose 6-phosphate product strongly suggests that BglT utilizes the same mechanism in the hydrolysis of thiodisaccharides as that of *O*-glycosidic linkages. Overall, there is no significant difference between the rates of hydrolysis of *O*- and *S*-glycosidic linkages as catalyzed by BglT. Hence, we have shown that BglT is the first glycosidase capable of efficiently cleaving unactivated thioglycosides.

Table 1: Summary of kinetic parameters for the hydrolysis of *O*- and *S*-glucosides by BglT.

Substrate	k_{cat} [s^{-1}]	K_M [μM]	k_{cat}/K_M [s^{-1} mM^{-1}]	K_i [μM]
O-pNPG6P[a]	1.9	41	46	–
S-pNPG6P[a]	1.6	41	39	–
O-pNPC6′P	0.017	1.0	17	1.0
S-pNPC6′P	0.072	8.0	9.0	5.0
O-C6′P[b]	0.61	69	8.8	70
S-C6′P	0.53	37	14	33

[a] Data obtained from Ref. [19]. [b] Data obtained from Ref. [24].



Scheme 3. Disaccharide substrates for BglT.

These findings clearly indicate a fundamental difference in the BglT mechanism compared to that of most glycosidases,

which cannot cleave unactivated thioglycosides. The efficient hydrolysis of thiodisaccharides therefore provides further supporting evidence for the novel glycosidase mechanism proposed for family 4 enzymes. Cleavage of such thioglycosides at rates comparable to those of their oxygen counterparts is quite reasonable for the anionic mechanism proposed but not for reactions via oxocarbenium-ion-like transition states. The majority of glycosidases do not hydrolyze thioglycosidic linkages, and those that have been reported to possess thioglycosidase activity only react with thioglycosides containing highly activated leaving groups, as clearly demonstrated by the Brønsted plot determined for thioglycoside hydrolysis in the case of *O*-GlcNAcase.[21] The cleavage of the thioglycosidic linkages by *O*-GlcNAcase does not rely on general acid catalysis, and there is substantial development of negative charge on the sulfur atom at the transition state.[21] Since the cleavage of the glycosidic linkage is not rate-limiting for BglT,[24] it is reasonable that substitution of the glycosidic oxygen with a sulfur atom does not significantly affect the overall rate.

On the basis of these results, it is clear that thioglycosides should not be employed in any inhibition strategies for family 4 enzymes whether this be for structural or mechanistic studies or as part of any biological control strategy. Indeed, the ability to cleave a thiodisaccharide could be a useful diagnostic of whether a new glycosidase belongs to family 4. Finally, these findings raise the question of whether *O*-C6′P is in fact the natural substrate for BglT, or whether this enzyme has evolved to cleave some other, as yet undiscovered, substrate. The locations of the genes encoding many GH4 enzymes within the PEP:PTS operon argues strongly that the disaccharide 6-phosphates are the natural substrates and that the facile cleavage of thioglycosides is just a circumstantial consequence of the mechanism utilized by the enzyme. However, the uncanny similarities between the mechanisms utilized by GH4 enzymes and by AdoHCyase suggests that other possibilities should be kept in mind.

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