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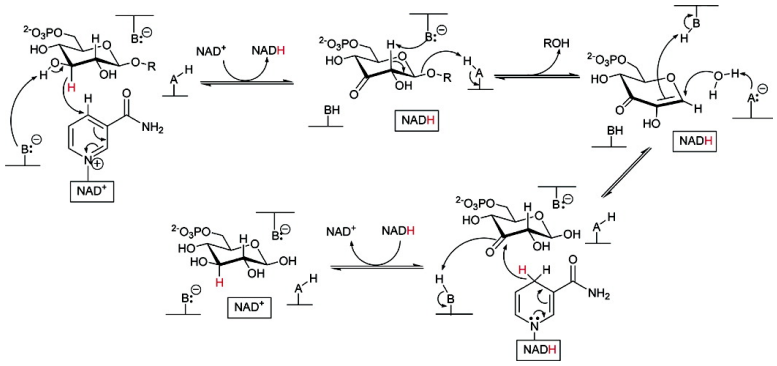

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An Unusual Mechanism of Glycoside Hydrolysis Involving Redox and Elimination Steps by a Family 4 β-Glycosidase from *Thermotoga maritima*

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Just over 50 years ago, the canonical mechanisms for enzymatic glycoside hydrolysis with retention or with inversion of sugar anomeric configuration were first published by Koshland,[1] and with only a few minor variations these mechanisms have stood the test of time.[2−4] Furthermore, the more recent classification of glycosidases into families on the basis of sequence similarities[5,6] has confirmed that, except as indicated below, enzymes within a family hydrolyze only glycosides of the same anomeric configuration and do so with the same stereochemical outcome.[7] The sole exceptions to this rule are the enzymes of family 4, some of which hydrolyze α-D-glycosides and some β-D-glycosides.[9−15] All members of this family to date are from bacterial sources, with many hydrolyzing only 6-phospho-glycosides.[10,12−15] Enzymes of this family are also unusual in requiring the cofactor NAD^+ for activity as well as a divalent metal ion, typically Mn^{2+}, and in many cases a reducing agent.[10,12,13,15−17] While a requirement for metal ions and reducing conditions is not that unusual for glycosidases, the requirement for NAD^+, which is not consumed,[10,12,17] and the accommodation of both α- and β-glycosidases within the same family are unprecedented and suggest a catalytic mechanism significantly different from those used by all other characterized glycoside hydrolases. Inspired by the mechanisms of NAD^+-dependent carbohydrate dehydratases involved in deoxy sugar biosynthesis,[18] a mechanism consistent with these observations is proposed for the 6-phospho-β-glucosidase BglT (E.C. 3.2.1.6) from *Thermotoga maritima* (see Figure 1). In support of this mechanism, we provide evidence including determination of stereochemical outcome, observation of solvent isotope exchange, measurement of primary kinetic isotope effects, and X-ray crystallographic analysis.

The mechanism proposed involves hydride abstraction at C3, thus oxidation of the 3-hydroxyl to a ketone. Such an oxidation acidifies the C2 proton, allowing deprotonation by an enzymatic base, accompanied by acid-catalyzed elimination of the glycosidic oxygen and formation of a 1,2-unsaturated intermediate. This α,β-unsaturated Michael-like acceptor then undergoes base-catalyzed attack by water to generate a 3-keto glucose derivative, which is finally reduced by the "on-board" NADH, returning the enzyme to its active form and completing the reaction cycle.

BglT was cloned and overexpressed in *Escherichia coli* (see Supporting Information). Kinetic studies with BglT, whose natural substrate is cellobiose 6′-phosphate, were performed with *p*-nitrophenyl 6-phospho-β-D-glucoside (pNPβG6P). Of the divalent metal ions tested, 1 mM Mn^{2+} was the best activator, followed by

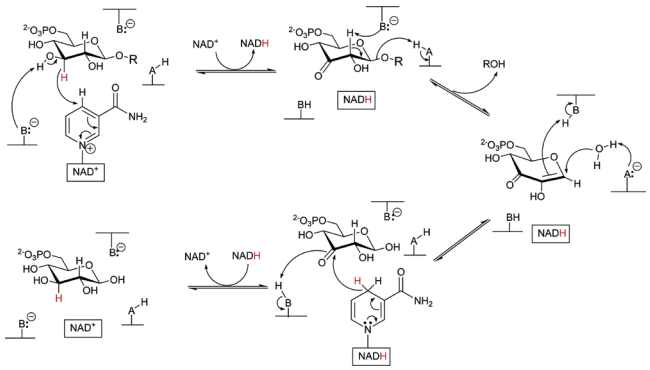
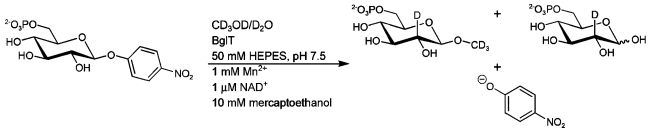


Figure 1. Proposed mechanism of BglT.

Mg^{2+} and Ca^{2+}, which provided 100-fold less activation. Four different nucleotides were investigated as potential activators (NAD^+, NADH, $NADP^+$, NADPH), of which only NAD^+ caused substantial rate increases: a 1 μM concentration was sufficient to provide full activity. Requirements for reducing agents were met by both dithiothreitol (DTT) and mercaptoethanol, of which mercaptoethanol was preferred since DTT forms heavy precipitates with Mn^{2+}. Under the optimized conditions (1 mM Mn^{2+}, 1 μM NAD^+, 10 mM mercaptoethanol), kinetic parameters of $k_{cat} = 1.9\ s^{-1}$ and $K_M = 41$ μM were measured for the hydrolysis of pNPβG6P by BglT. In contrast to what had been seen for other family 4 glycosidases such as MalH, only pNPβG6P, and not its α-anomer *p*-nitrophenyl 6-phospho-α-D-glucoside (pNPαG6P), was a substrate.[19]

A mechanistic signature of glycosidases is the stereochemical outcome of the reaction they catalyze. Direct analysis by ^1H NMR was rendered problematic by the requirement for the paramagnetic Mn^{2+} cation for optimal activity, which broadens signals. The very rapid mutarotation of the product glucose 6-phosphate (G6P)[20] also made "stopped" analysis involving Mn^{2+} removal impractical. A solution was found in carrying out reactions in the presence of 5 M CD_3OD such that a mixture of the stereochemically stable trideuteriomethyl 6-phospho-glucoside and the hydrolysis product is formed. Fortunately, BglT is active under such conditions. ^1H NMR analysis of such reaction mixtures after removal of enzyme and Mn^{2+} revealed a mixture of G6P and the β-anomer of trideuteriomethyl 6-phospho-glucoside. The α-anomer was not



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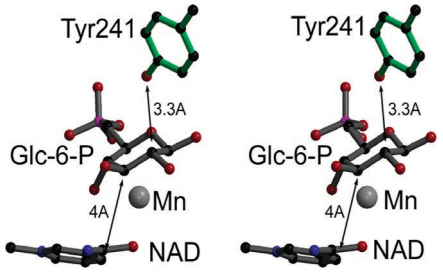
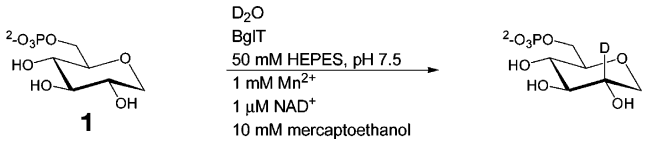


Figure 2. Active center of the 6-phospho-β-glucosidase BglT as revealed by the 2.55 Å structure determination in the presence of Mn^{2+}, G6P, and NAD^+ (PDB code 1up6). The figure shown is in divergent (wall-eyed) stereo and was drawn with the BOBSCRIPT program.[23]

formed, indicating that BglT is a retaining glycosidase and implying the same stereochemical outcome for all family 4 enzymes.

A surprising finding was that the C2 proton of the methyl glycoside product had fully exchanged with solvent D_2O/CD_3OD, as revealed by the observation of a singlet for the anomeric proton in the ^1H NMR spectrum. The same exchange was observed when the reaction was carried out in D_2O buffer in the absence of methanol, while no such exchange was observed when pNPβG6P or G6P was incubated in D_2O buffer for similar time periods in the absence of enzyme. Exchange of the C2 proton is therefore a direct consequence of the catalytic mechanism and is fully consistent with activation by the adjacent ketone at C3. Confirmation of this came from the finding that solvent exchange at C2 also occurs on 1,5-anhydroglucitol 6-phosphate,[21] **1**, when incubated with BglT in D_2O buffer. The absence of a leaving group at the anomeric center and the inability of the substrate to ring-open remove two possible side reactions that could lead to exchange processes, thereby directly supporting a mechanism involving oxidation of the 3-hydroxyl.



Further evidence in support of the proposed oxidation at O3 was obtained via kinetic isotope effect (KIE) measurements using the substrate pNPβG6P and the corresponding 3-[^{2}H] substrate, *p*-nitrophenyl 3-[^{2}H]-6-phospho-β-D-glucoside. KIEs for the 3-[^{2}H] substrate were found to be $k_H/k_D = 1.63 \pm 0.01$ (at 15 K_M) and $(k_{cat}/K_M)_H/(k_{cat}/K_M)_D = 1.91 \pm 0.03$ (at 0.15 K_M). Small, but significant, primary KIEs of $k_H/k_D = 1.84 \pm 0.02$ and $(k_{cat}/K_M)_H/(k_{cat}/K_M)_D = 2.03 \pm 0.01$ were similarly measured for *p*-nitrophenyl 2-[^{2}H]-6-phospho-β-D-glucoside, consistent with the bond cleavage event at that center as well. These small primary KIEs indicate that the hydride elimination and proton abstraction steps are both partially rate-limiting. They are extremely hard to reconcile with any other mechanism and thus confirm that cleavage of the C3−H3 linkage occurs during a rate-limiting step of the reaction mechanism.

An excellent structural foundation for the proposed mechanism was secured when the three-dimensional structure of BglT in the presence of product G6P was solved to 2.55 Å resolution (with 8-fold noncrystallographic symmetry averaging), PDB code 1up6. As shown in Figure 2, the NAD^+ cofactor is located in the active site, directly beneath C3 at a distance of 4 Å, in an excellent position for hydride abstraction. Arrayed around the substrate are a series of side chains that likely play important roles in catalysis, with the Mn^{2+} coordinating to the substrate 2- and 3-hydroxyls and presumably stabilizing the α-hydroxy ketone intermediate and its enolate anion. Tyr241, potentially activated by a nearby carboxylic acid residue, appears to be the catalytic base. Similarly, a Tyr residue, deprotonated by Glu through the participation of a His residue, has been shown to function as a general base in a chrondroitin AC lyase, which also utilizes an elimination mechanism.[22]

Interestingly, a crystal structure of an inactive family 4 α-glucosidase, AglA, was solved by Lodge et al.,[11] revealing pronounced structural similarities with α-hydroxy organic acid dehydrogenases, much as is also the case with the 6-phospho-α-glucosidase, GlvA, from *Bacillus subtilis* (PDB code 1nrh). BglT has a similar structure, with the NAD^+ located in an equivalent position with respect to the substrates in each case. Also similar are the structures of the sugar dehydratases, such as RmlB, the *d*TDP-D-glucose 4,6-dehydratase involved in the L-rhamnose biosynthetic pathway.[18,24] This enzyme catalyzes the elimination of water across the 4,6-bond and is activated for this role by transient oxidation of O3 by an "on-board" NAD^+ in much the same manner as the first steps of BglT.[18] Current data suggest that family 4 glycosidases and dehydratases, two seemingly distinct classes of enzymes, may have evolved to operate through similar mechanistic pathways, possibly from similar progenitors.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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