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Catalytic properties of a mutant β-galactosidase from *Xanthomonas manihotis* engineered to synthesize galactosyl-thio-β-1,3 and -β-1,4-glycosides

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Abstract The identity of the acid/base catalyst of the Family 35 β-galactosidases from *Xanthomonas manihotis* (BgaX) has been confirmed as Glu184 by kinetic analysis of mutants modified at that position. The Glu184Ala mutant of BgaX is shown to function as an efficient thioglycoligase, which synthesises thiogalactosides with linkages to the 3 and 4 positions of glucosides and galactosides in high (>80%) yields. Kinetic analysis of the thioglycoligase reveals glycosyl donor K_m values of 1.5–21 μM and glycosyl acceptor K_m values from 180 to 500 μM. This mutant should be a valuable catalyst for the synthesis of metabolically stable analogues of this important glycosidic linkage.

Keywords: Glycoside hydrolase Family 35; *Xanthomonas manihotis* β-galactosidase; Acid/base catalyst; Thioglycoligase; Thioglycosides

1. Introduction

Due to the important roles of oligosaccharides and glycans in many biological processes in cells [1], the naturally occurring oligosaccharides and their analogues are of particular interest as carbohydrate-based therapeutics [2]. However, the application of carbohydrate-based therapeutics may be limited, in some cases, as a consequence of their susceptibility to enzymatic hydrolysis by endogenous glycosidases [3]. One strategy towards circumventing this problem is to replace the interglycosidic oxygen atom with a sulfur atom to give sulfur-linked oligosaccharides [4,5]. Glycosidase-resistant thioglycoside analogues of the original oligosaccharides are therefore attractive candidates as potential therapeutics, as stable antigens, and as biochemical probes due to their much lower rates of chemical and enzymatic hydrolysis and their similar conformations to those of their natural *O*-linked counterparts [6]. A number of successful chemical syntheses of thio-oligosaccharides have been reported [7–9], but control of stereochemistry remains challenging. Recently, enzymatic strategies have been reported using mutant forms of glycosidases: thioglycoligases [10] and thioglycosynthases [11] as well as, but less efficiently to date, glycosyltransferases [12].

Thioglycoligases are mutant enzymes derived from retaining glycosidases in which the acid/base carboxylic acid residue has been replaced by an amino acid that has no negative charge (Fig. 1). When these mutants are incubated with substrates bearing a good leaving group, such as dinitrophenol (DNP) or fluoride, they relatively rapidly form a covalent glycosyl-enzyme intermediate because they do not need assistance from the acid catalyst. Since the mutants have no general base catalyst, the rates of transglycosylation to normal acceptors such as water and hydroxyl-containing sugars are extremely low. However, transfer occurs efficiently to acceptors bearing a suitably-positioned thiol since the thiol group is much more nucleophilic and requires no general base catalytic assistance. The thioglycoligase strategy has been applied recently in the synthesis of thio-linked inhibitors for use as structural probes [13] and in the modification of glycans on the surface of a model glycoprotein [14].

A class of glycosidic linkages that is of particular interest, but which has not yet addressed by glycosynthase [15] or thioglycoligase strategies, is that of the Gal-β-1,3-GlcNAc and Gal-β-1,3-GalNAc linkages found in gangliosides and glycoproteins. While β-galactosidases are found in glycoside hydrolase (GH) Families 1, 2, 35, and 42, only those in Family 35 have specificities directed toward Gal-β-1,3-linkages. This family contains the human lysosomal β-galactosidases responsible for catabolism of such glycoconjugates and deficiencies in this enzyme arising from mutations lead to the neurological disorders GM1-gangliosidosis and Morquio B. syndrome [16]. Through the use of mechanism-based inactivators that result in trapping of a glycosyl-enzyme intermediate, the catalytic nucleophile of this enzyme was identified as Glu268 [17]. Labeling of the equivalent residue, Glu260, in the homologous bacterial enzyme from *Xanthomonas manihotis* confirmed the nucleophilic role of this residue [18]. No information was available on the identity of the acid/base catalyst until the relatively recent solution of the three-dimensional structure of the Family 35 β-galactosidase from *Penicillium* sp. by X-ray crystallography [19]. On the basis of a product complex with galactose,

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Abbreviations: DNP, 2,4-dinitrophenyl; PNP, 4-nitrophenyl; BgaX, β-galactosidase from *Xanthomonas manihotis*; Abg, β-glucosidase from *Agrobacterium* sp.; GH, glycoside hydrolase; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; ESI, electrospray ionization; DNPGal, 3,4-dinitrophenyl β-D-galactopyranoside; PNPGal, 4-nitrophenyl β-D-galactopyranoside; PNP4SGlc, 4-nitrophenyl 4-deoxy-4-thio-β-D-glucopyranoside; PNP3SGlc, 4-nitrophenyl 3-deoxy-3-thio-β-D-glucopyranoside; PNP6SGlc, 4-nitrophenyl 6-deoxy-6-thio-β-D-glucopyranoside; PNP4SGal, 4-nitrophenyl 4-deoxy-4-thio-β-D-galactopyranoside; PNP3SGal, 4-nitrophenyl 3-deoxy-3-thio-β-D-galactopyranoside

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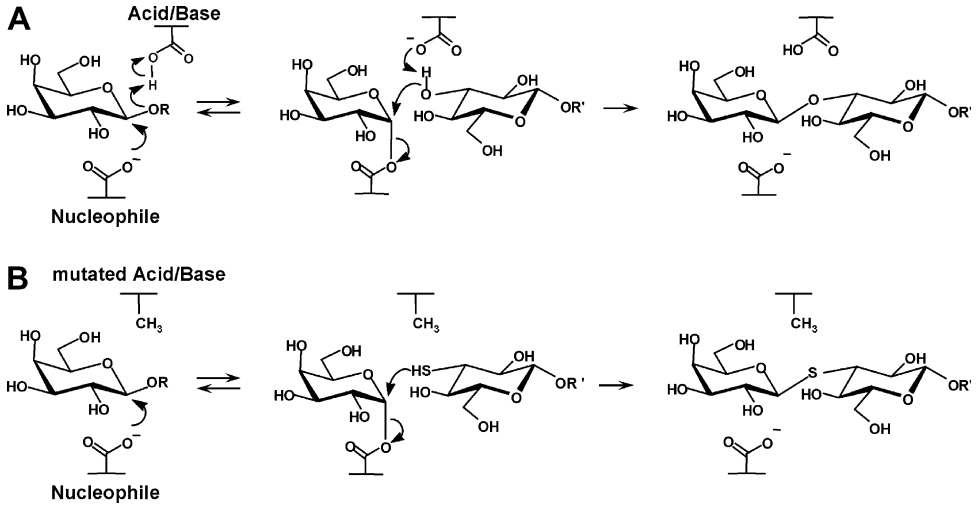

Fig. 1. Mechanisms of transglycosylation by retaining galactosidases (A) and thiogalactoligases (B).

Glu200 was identified as the putative acid/base catalyst. However, there has been no confirmation of this proposed role by kinetic analysis of mutants. The β-galactosidase from *X. manihotis* (BgaX) is a good test system with which to confirm this assignment and to assess the potential for generation of thioglycoligases of the desired specificity. The wild type enzyme has been shown previously to synthesise Gal-β-1,3-GlcNAc linkages via transglycosylation, albeit in rather low yields [20,21], though attempts to generate a glycosynthase from BgaX have been unsuccessful to date (Blanchard, J. and Withers, S.G., unpublished data).

Herein, we describe the confirmation of Glu184 as the acid/base catalyst in BgaX, analogous to Glu200 in *Penicillium* sp. β-galactosidase, by azide rescue analysis with the Glu184Ala mutant. We demonstrate that this mutant functions as an efficient thioglycoligase capable of synthesizing both β-1,3- and β-1,4-thioglycosidic linkages, depending on the position of the thiol-group in the acceptor. Detailed kinetic analyses revealed low K_m values for both glycosyl donors and thio-sugar acceptors, and substantial preference for transfer to the 3-position of gluco-configured acceptors.

2. Materials and methods

2.1. Materials and general analysis

All chemicals were obtained from the Sigma Chemical Co. unless otherwise specified. *Pwo* polymerase was purchased from Roche and restriction enzymes from Fermentas (Germany). 4-Nitrophenyl 4-deoxy-4-thio-β-D-glucopyranoside (PNP4SGlc), and 4-nitrophenyl 4-deoxy-4-thio-β-D-galactopyranoside (PNP4SGal) were synthesized according to literature procedures [10]. The synthetic details of the preparation of 4-nitrophenyl 6-deoxy-6-thio-β-D-glucopyranoside (PNP6SGlc), 4-nitrophenyl 3-deoxy-3-thio-β-D-glucopyranoside (PNP3SGlc), and 4-nitrophenyl 3-deoxy-3-thio-β-D-galactopyranoside (PNP3SGal) will be published elsewhere. All ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz using a Bruker AV-400 spectrometer. Mass spectra of small molecules were recorded using a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, Ont., Canada) equipped with an electrospray ionization (ESI) ion source. Thin layer chromatography (TLC) was performed on aluminum-backed sheets of silica gel $60F_{254}$ (Merck) of thickness 0.2 mm. The plates were visualized using UV light (254 nm) and/or by exposure to 10% sulfuric acid in methanol followed by charring.

2.2. Construction of BgaX-E184A and E184Q mutants

The mutations of the acid/base residue of BgaX were introduced using a mega primer PCR method. The gene encoding the 6x histidine tagged BgaX obtained by digestion of pTUG10N18/β-Gal [18] with *Nco*I and *Hin*dIII was subcloned into a pET28a vector (Novagen). The resulting plasmid, namely pET28BgaX$(His)_6$, was used as the template for the mutation of BgaX. The front ends of the genes for BgaX-E184A and BgaX-E184Q were first amplified using a T7 promoter primer and the following BgaX-E184A-rev primer (5′-GTCGGCGTAGGAGCCGTAGGCGTTCTCGAC-3′) and BgaX-E184Q-rev primer (5′-GTCGGCGTAGGAGCCGTACTGGTTCTCGAC-3′). The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen) and then used as megaprimers to obtain the full length genes with the T7 terminator primer. The final PCR products were digested with *Nco*I and *Hin*dIII, then extracted from the agarose gel using a QIAquick Gel Extraction Kit and ligated with pET28a that had been digested with the corresponding restriction enzymes. The resulting plasmids were designated pET28BgaX-E184A$(His)_6$ and pET28BgaX-E184Q$(His)_6$, respectively.

2.3. Expression and purification of BgaX-E184A mutant

The plasmids containing genes for BgaX mutants were transformed into *Escherichia coli*, and the recombinant *E. coli* was cultured for 6 h at 37 °C in Luria–Bertani medium containing 20 μg/mL of kanamycin, followed by overnight IPTG induction (0.2 mM) at 30 °C. The crude enzyme solutions were prepared by lysis of the harvested cells using Bugbuster™ solution (Novagen). The crude enzyme solutions were centrifuged at $10000 \times g$ for 30 min and the supernatants were loaded onto a HisTrap™ FF column (Amersham Biosciences). BgaX mutants were eluted using a step-gradient of 10 mM → 20 mM → 250 mM imidazole in 20 mM Tris–HCl buffer (pH 8.0) containing 500 mM NaCl. For further purification, ammonium sulfate was added directly to the fractions containing BgaX mutants until the final concentration of ammonium sulfate was 2 M, then the enzyme solutions were subjected to hydrophobic interaction chromatography using a HiTrap™ Phenyl HP column (Amersham Biosciences). BgaX mutants were eluted with a linear gradient of 2 M → 0 M ammonium sulfate in 20 mM Tris–HCl buffer (pH 8.0). The pure enzyme fractions were combined, dialyzed, and then concentrated using an Amicon Ultra-4 filter unit (10000 Da, cut-off, Millipore). Concentrations of enzyme solution were quantified by the Bradford method using bovine serum albumin as a standard [22].

2.4. Hydrolysis and thioglycosylation kinetics

All kinetic studies were performed at 30 °C in pH 7.0, 100 mM phosphate buffer. Twenty microliters of BgaX-E184A was added to 100 μL of buffer containing either 3,4-dinitrophenyl β-D-galactopyranoside (DNPGal) or 4-nitrophenyl β-D-galactopyranoside (PNPGal) as donors and thio-sugar acceptors for transglycosylation. Hydrolysis of