Directed evolution of a β-glycosidase from Agrobacterium sp. to enhance its glycosynthase activity toward C3-modified donor sugars

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Glycans bearing modified hydroxy groups are common in biology but because these modifications are added after assembly, enzymes are not available for the transfer and coupling of hydroxyl-modified monosaccharide units. Access to such enzymes could be valuable, particularly if they can also introduce ‘bio-orthogonal tags’. Glycosynthases, mutant glycosidases that synthesize glycosides using glycosyl fluoride donors, are a promising starting point for creation of such enzymes through directed evolution. Inspection of the active site of a homology model of the GH1 Agrobacterium sp. β-glycosidase, which has both glucosidase and galactosidase activity, identified Q24, H125, W126, W404, E411 and W412 as amino acids that constrain binding around the 3-OH group, suggesting these residues as targets for mutation to generate an enzyme capable of handling 3-O-methylated sugars. Site-directed saturation mutagenesis at these positions within the wild-type β-glycosidase gene and screening via an on-plate assay yielded two mutants (Q24S/W404L and Q24N/W404N) with an improved ability to hydrolyze 4-nitrophenyl 3-O-methyl-β-D-galactopyranoside (3-MeOGal-pNP). Translation of these mutations into the evolved glycosynthase derived from the same glucosidase (2F6) yielded glycosynthases (AbgSL-T and AbgNN-T, where T denotes transferase) capable of forming 3-O-methylated glycosides on multi-milligram scales at rates approximately 5 and 40 times greater, respectively, than the parent glycosynthase.

Keywords: directed evolution/glycosidase/glycosynthase/site-saturation mutagenesis/3-O-methyl glucose

Introduction

Glycosides play a key role in various biological processes. They are utilized in energy metabolism, serve as structural components, and are involved in the recognition of glycoconjugates, which have a range of functions (Weigel and Yik, 2002; Tumbale et al., 2008; Shim et al., 2009). For example, they assist the folding of glycoproteins, play important roles in immune receptors (Dwek, 1996; Chan et al., 2009), modulate the degradation of glycoproteins by proteases and assist in the delivery of some glycoconjugates to their targets via binding to lectins (Varki, 1993). Synthetic glycans and glycoconjugates are valuable tools in glycobiology and can be prepared by chemical and enzymatic methods. Of these approaches, chemical synthesis has the greatest flexibility in terms of structural modifications, but it is limited in terms of polypeptide size and scale (Gamblin et al., 2009; Huang et al., 2009; Rich and Withers, 2009). Enzymatic methods are not restricted by polypeptide length, and they utilize relatively moderate reaction conditions, but can have limited applications to unnatural sugars (Perugino et al., 2004; Rich and Withers, 2009).

There are two general enzymatic methods for forming glycosidic linkages. The most common is the use of glycosyltransferases (GTs), which catalyze the attachment of sugar moieties to acceptor molecules (Williams and Thorson, 2009; Hansen et al., 2010). Usage of GTs can be limited by such factors as the cost of the nucleotide diphosphosugar donor and poor availability because of solubility and expression problems (Hancock et al., 2005). The second method for making glycosidic linkages relies on the transglycosylation activity of glycosidases, though this approach can be limited by competing hydrolysis (Shaikh and Withers, 2008). To solve this problem, mutant glycosidases capable of carrying out transglycosylation but not hydrolysis have been developed (Mackenzie et al., 1998; Perugino et al., 2004). These nucleophile mutants, or glycosynthases, can exhibit high product yields, and their substrates (glycosyl fluorides) are relatively inexpensive and easy to prepare (Hancock et al., 2005; Shaikh and Withers, 2008). They have been used to prepare a wide range of glycosomes, from oligo and polysaccharides through to glycoconjugates such as glycolipids, glycosylated aromatics and glycoproteins.

By application of directed evolution methodologies, the activities of glycosynthases have been improved for their ‘natural’ sugars and some progress has been made with broadening of glycone specificivity (Drone et al., 2005; Feng et al., 2005; Hancock et al., 2005). However, little attention has been paid to the engineering of enzymes to handle either naturally modified sugars or ones bearing artificial tags for subsequent reactions. Examples of modified sugars include the 3-O-methyl hexose derivatives found in various snails and seaweeds as well as the methylated sugars that cap the surface polysaccharides of Mycobacteria. Such methylation is typically added using S-adenosylmethionine after glycan assembly (Antonov et al., 2009; Muthana et al., 2012). In this report we describe our first such studies on the Agrobacterium sp. β-glycosidase which has both β-glucosidase and β-galactosidase activity. This enzyme (Abg), along with its glycosynthase mutant (2F6, which bears the following mutations: A19T, Q248R, E358G and M407V),
Experimental procedures

Construction of p6xHTKNdAbg

The abg gene from pGSVIII (Kim et al., 2004) was excised by digestion with NdeI and XhoI then subcloned into pTKNd119 bearing the Bacillus licheniformis maltogenic amylase promoter and a C-terminal hexahistidine tag, (Kim FH, QH, RW, RE and QE) were obtained using the primer method was used to create library A (A). In library A, combination mutants containing two to six mutations per gene were prepared using PCR with five mutated DNA fragments (A).

Site-directed saturation mutagenesis and the construction of mutant libraries

As shown in Fig. 1, four saturation mutagenesis libraries (at Glu24, His125/Trp126, Trp404 and Gln411/Trp412 of Abg) were created in concert (library A) and individually (library B), and the mutagenic primers are summarized in Table I.

The six polymerase chain reaction (PCR) fragments (FQ, FH, QH, RW, RE and QE) were obtained using the abg gene in pET29bAbg as template (Mayer et al., 2000), and the corresponding forward and reverse primers (pairs) listed in Table I. PCR conditions: 1 μM for each of the corresponding forward and reverse primers, 0.25 mM dNTPs, 20 ng of pET29bAbg, and 2 U of Pfu polymerase (Fermentas, Burlington, Canada) in 50 μl of 1 × Pfu polymerase buffer. Thirty cycles of PCR (30 s at 95°C, 30 s at 55°C, and 30 s at 72°C) were carried out in a thermal cycler (GeneAmp PCR system 2400; Perkin Elmer Life Sciences, Waltham, MA, USA). In the case of the QE fragment (1.2 kbp), the elongation time was 60 s.

Construction of library A mutants

Approximately 100 μg of these five PCR products (FQ, QH, RW, RE and QE) were mixed for primerless PCR (20 cycles of 30 s at 95°C, 60 s at 55°C and 60 s at 72°C). In this reaction, each fragment acted as both primer and template. The resulting products were subcloned into pTKNd119 using NdeI and XhoI, respectively.

Construction of library B mutants

The full length mutagenic products were obtained with 20 ng pET29bAbg as template, 1 μM of each of the forward mega primers, FQ or FH, and reverse Abg-Term; or, forward T7-Pro and reverse mega primers RW or RE using 25 cycles of PCR (30 s at 95°C, 30 s at 55°C, and 60 s at 72°C). The resulting 1.4 kbp mutagenic PCR products were double digested with NdeI and XhoI, and cloned into pTKNd119.

The mutant libraries were transformed into R1360 (Schneitz and Rak, 1992) chemically competent Escherichia coli cells. Transformants were incubated on Luria–Bertani agar media containing 20 μg/ml kanamycin (LBkan agar).

Site-directed mutagenesis

To introduce effective mutations into the gene of 2F6, an Abg glycosynthase mutant with high catalytic activity (Kim et al., 2004), a QuikChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA) was employed. Four sets of primers were used: Q24S, Q24N, W404L and W404N (Table I).

Screening of the mutant library

For the rapid detection of positive clones, our previous screening method (Kim et al., 2004; Müllegger et al., 2005) was modified. After transformation of the mutant library, cells were incubated on agar plates at 37°C overnight and transferred to Hybond-N membranes (137 mm; Amersham Biosciences, Piscataway, NJ, USA). In order to lyse the cell wall, each membrane was soaked in 600 μl of Tris–HCl buffer (50 mM, pH 8.0) containing 0.1% (w/v) of polymyxin B sulfate (Sigma, St. Louis, MO, USA) and incubated at 37°C for 60 min (Van Poucke and Nelis, 2000). Next, 0.5 ml of substrate solution (1 mM 4-methylumbelliferyl 3-O-methyl-β-D-galactoside (3-MeOGal-MU) in 50 mM Tris–HCl buffer, pH 8.0) was added to each membrane. During incubation of the membranes at room temperature, fluorescent signals caused by mutant enzyme activity were monitored under UV light (395 nm) and the most positive clones (10–15 clones per 1000 colonies) chosen from master plates. The clones selected in the first screen were inoculated individually into 96-deep well culture tubes, each containing 200 μl of LBkan, and grown in a shaking incubator (250 rpm) at 37°C overnight. The cell density of each culture was measured based on the optical density at 600 nm using a spectrophotometer (SPECTRAMax Plus; Molecular Devices Corp., Sunnyvale, CA, USA). Approximately 70 μl of each culture was transferred to a 96-well plate; the remaining volume was...
Table I. Oligonucleotides used to generate the mutant Abg and Abg glycosynthases

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7-Pro</td>
<td>5'-taatacgactcaaatagg-3'</td>
<td>Forward, FO</td>
</tr>
<tr>
<td>Abg-Term</td>
<td>5'-ggctcggagcaggccggaagcggaatcctggccaaggtaggtttggccggaagcctggagcgatccgctgc-3'</td>
<td>Reverse, RE</td>
</tr>
<tr>
<td>7Q4X-F</td>
<td>5'-ctctctcgcggtacggcttttggaggtgtggagggggtttgtgagg -3'</td>
<td>Forward, QE</td>
</tr>
<tr>
<td>7Q4X-R</td>
<td>5'-gggctgggagctggagggggtttgtgagg -3'</td>
<td>Reverse, FQ</td>
</tr>
<tr>
<td>H12XW126X-R</td>
<td>5'-cagatgcgggctgggggggtttgtgagg -3'</td>
<td>Reverse, RE</td>
</tr>
<tr>
<td>W404X-F</td>
<td>5'-gtctgggagctggagggggtttgtgagg -3'</td>
<td>Reverse, QE</td>
</tr>
<tr>
<td>E141XX142X-F</td>
<td>5'-ctctctcgcggtacggcttttggaggtgtggagggggtttgtgagg -3'</td>
<td>Forward, R24N</td>
</tr>
<tr>
<td>E141XX142X-R</td>
<td>5'-ctctctcgcggtacggcttttggaggtgtggagggggtttgtgagg -3'</td>
<td>Reverse, Q24N</td>
</tr>
<tr>
<td>Q24X-F</td>
<td>5'-ggcgtctgggagctggagggggtttgtgagg -3'</td>
<td>Reverse, Q24N</td>
</tr>
<tr>
<td>Q24X-R</td>
<td>5'-ggcgtctgggagctggagggggtttgtgagg -3'</td>
<td>Reverse, Q24N</td>
</tr>
<tr>
<td>Q24S-F</td>
<td>5'-ggcgtctgggagctggagggggtttgtgagg -3'</td>
<td>Reverse, Q24N</td>
</tr>
<tr>
<td>Q24S-R</td>
<td>5'-ggcgtctgggagctggagggggtttgtgagg -3'</td>
<td>Reverse, Q24N</td>
</tr>
<tr>
<td>W404L-F</td>
<td>5'-gtctgggagctggagggggtttgtgagg -3'</td>
<td>Reverse, Q24N</td>
</tr>
<tr>
<td>W404L-R</td>
<td>5'-gtctgggagctggagggggtttgtgagg -3'</td>
<td>Reverse, Q24N</td>
</tr>
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<td>W404N-F</td>
<td>5'-gtctgggagctggagggggtttgtgagg -3'</td>
<td>Reverse, Q24N</td>
</tr>
<tr>
<td>W404N-R</td>
<td>5'-gtctgggagctggagggggtttgtgagg -3'</td>
<td>Reverse, Q24N</td>
</tr>
</tbody>
</table>

Mutation sites are underlined.

stored at 4°C. Cell wall lysis was achieved using an equal amount (70 μl) of BugBuster (Novagen, San Diego, CA, USA) with incubation for 30 min at room temperature. Next, the crude cell extracts were mixed with 140 μl of assay solution (0.5 mM 3-MeOGal-MU in 50 mM Tris–HCl buffer, pH 8.0). The release of methylumbelliferone was monitored by a Wallac 1420 VICTOR2 (Perkin Elmer) plate reader. Mutant enzymes with higher activity for 3-MeOGal-MU than wild-type Abg were chosen, purified, and analyzed further.

Purification of Abg and its mutants

Wild-type and mutant Abg enzymes were purified from E. coli R1360 cells harboring the corresponding genes on pTKNd119 by affinity chromatography using nickel-nitrilotriacetic acid agarose (QIAGEN) as described previously (Mayer et al., 2000). The purified enzymes were desalted and concentrated using an Ultra-4 filter unit (10 000 Da molecular weight cut-off; Millipore, Billerica, MA, USA). The protein concentration was measured at 280 nm with the appropriate extinction coefficient (Pace et al., 1995).

Kinetic analysis of the glycosidases and glycosynthases

Kinetic analysis of glycosidase activity was carried out at 37°C in Na-phosphate buffer (50 mM, pH 7.0) containing 0.1% (w/v) bovine serum albumin, the appropriate 4-nitrophenyl glycoside and various concentrations of enzyme (0.06–6 μM). A Cary 4000 UV/visible spectrophotometer (Varian, Santa Clara, CA, USA) was used to monitor the release of p-nitrophenol (pNP) by measuring the change in absorbance at 400 nm using an extinction coefficient of 7500 cm−1 M−1. Kinetic studies on the glycosynthase were performed with a fluoride electrode interfaced with an Accumet 925 pH/ion meter (Fischer Scientific, Pittsburgh, PA, USA) to monitor the release of fluoride at 30°C (Kim et al., 2004). Values of Km and kcat for C3-modified glycosyl fluorides were determined at a fixed (19.5 mM) concentration of acceptor sugar (4-nitrophenyl β-cellobioside, pNP-cellobioside) and varied (20–330 mM) donor sugar. In cases where a high Km prevented the determination of individual Michaelis–Menten parameters, the apparent kcat/Km value was determined from the slope of v versus [S] at low substrate concentrations. The kinetic parameters of Abg and the mutants were determined by direct fitting to the Michaelis–Menten equation using GraFit (version 5.0; Erithacus Software Ltd., Surrey, UK).

Oligonucleotide synthesis, DNA sequencing and glycoside product analysis

DNA sequence analysis and PCR primer synthesis were performed by the Nucleic Acid and Peptides Service Unit of the Biotechnology Laboratory at the University of British Columbia. Mass spectrometric (MS) and nuclear magnetic resonance (NMR) analysis of the glycoside product was performed in the Spectroscopic facilities of the Department of Chemistry at the University of British Columbia.

Model structure

The structures of Abg and its mutants were determined using SWISS-MODEL (ver. 3.51) at the ExPASy server (Peitsch, 1996). Previously reported three-dimensional β-glucosidase structures were employed as templates. The RCSB Protein Data Bank entries were 1BGA (Sanz-Aparicio et al., 1998), 1QOX (Hakulinen et al., 2000), 1GOW (Aguilar et al., 1997) and 1QVB (Chi et al., 1999). The structures were visualized using PyMol ver. 0.99 and Swiss-PDB Viewer ver. 4.0.

Results

Previous kinetic analyses of Abg using a range of glycoside substrates (Day and Withers, 1986; Kempton and Withers, 1992; Namchuk and Withers, 1995) have revealed a relatively tight specificity for the 3-hydroxyl group. Replacement by hydrogen (deoxy) or fluorine (deoxyfluoro) resulted in reduction of the rate constant (kcat/Km) by 17- and 11-fold respectively, thus hydrogen bonds at this position contribute 1.5–1.8 kcal.mol−1 toward stabilization of the transition state. Attempted hydrolysis of 4-nitrophenyl 3-O-methyl-β-p-galactopyranoside (3-MeOGal-pNP) by wild-type Abg revealed an extremely slow reaction with no sign of substrate saturation, thus a very high Km value. The kcat/Km value of 0.035 min−1 mM−1 determined is some 105-fold lower than that for pNP-Gal (Table II). Although part of this rate...
Table II. Comparison of the hydrolytic kinetic parameters of Abg and its mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m$ (nM)</th>
<th>$k_{cat}/K_m$ (min$^{-1}$ nM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abg</td>
<td>pNP-Glc</td>
<td>6170 ± 294</td>
<td>0.06 ± 0.0081</td>
<td>98 800</td>
</tr>
<tr>
<td></td>
<td>pNP-Gal</td>
<td>11 523 ± 1080</td>
<td>3.1 ± 0.71</td>
<td>3703</td>
</tr>
<tr>
<td></td>
<td>3-MeOGal-pNP</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AbgSL</td>
<td>pNP-Glc</td>
<td>11 ± 0.36</td>
<td>1.58 ± 0.15</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>pNP-Gal</td>
<td>4.9 ± 0.16</td>
<td>18.5 ± 1.46</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>3-MeOGal-pNP</td>
<td>2.9 ± 0.25</td>
<td>17.0 ± 3.34</td>
<td>0.17</td>
</tr>
<tr>
<td>AbgNN</td>
<td>pNP-Glc</td>
<td>20 ± 0.66</td>
<td>3.5 ± 0.36</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>pNP-Gal</td>
<td>7.9 ± 0.38</td>
<td>23.4 ± 2.53</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>3-MeOGal-pNP</td>
<td>6.1 ± 0.63</td>
<td>26.1 ± 3.15</td>
<td>0.23</td>
</tr>
</tbody>
</table>

ND, not determined.

Fig. 2. Model structure of α-glucosyl fluoride bound to wild-type Abg showing interactions around the substrate C3–OH group. The side chains of the residues are labeled, with carbon atoms shown in gray; oxygen in red; nitrogen in blue and the substrate fluorine in light blue.

reduction is likely due to the loss of a hydrogen bonding interaction with the OH moiety, the majority must be due to deleterious steric interactions.

Inspection of a homology model of the active site generated using data from homologous enzymes (Protein Data Bank code 2CET, 1QOX and 1QVB) revealed that the 3-OH group is surrounded by six amino acids within 5 Å (Fig. 2). Among these, five amino acids (Q24, H125, W404, E411 and W412) are highly conserved in β-glucosidases of the GH1 family (Corbett et al., 2001; Marana et al., 2002). Two of these, Gln24 and Trp412, interact directly with the equatorial 3-OH group of the substrate.

A logical approach toward generating diversity around this position within the active site was therefore to create saturation mutagenesis libraries representing each of these positions. Fortunately, two pairs of these sites are immediate neighbours (H125/W126: E411/W412) making the construction of saturation mutagenesis libraries simpler. Two approaches were employed to this end. In the first approach (library A), mega primers FQ, RW, QH, RE and QE were first prepared by PCR using primers listed in Table I. These were then mixed and subjected to primerless PCR to assemble the collection of mutagenized genes. Sequencing revealed that every clone had two to six mutations at the expected sites. However, as fragments QE and RW possessed parental sequences at the target sites, clones covering all six sites were rare. To overcome this problem, library B was generated by PCR amplification of each of the four mega primers with their corresponding matching primer (Abg-Term or T7-Pro). Sequence analysis showed one or two mutations at each site (Fig. 1B).

The ultimate objective of this work is to generate glycosynthases capable of adding sugar residues modified at C3 onto acceptors and indeed we have developed a useful direct screen for Abg glycosynthase involving an on-plate coupled assay in which a cellulase that hydrolyses the fluorogenic cellulobiose products, but not the fluorogenic glucoside substrate, was co-expressed. However, the coupling enzyme would not accept the modified products formed in this study, eliminating this approach. It was therefore decided to first perform mutagenesis of the wild-type enzyme as screening for the hydrolysis of chromogenic substrates is considerably simpler than screening for glycosynthase activity. Further, it would be useful to also have access to the modified glycosidases themselves as tools for removal of naturally occurring 3-O-methyl-modified sugars. As the specificities of glycosynthases derived to date have largely mirrored the specificities of the hydrolases from which they were derived it was highly likely that transfer of the successful mutations identified into our best evolved glycosynthase framework (2F6) would provide the desired glycosynthase.

The screening protocol thus involved growth on agar plates at 37°C overnight, then transfer onto membranes, which were soaked in buffer containing polymyxin to lyse cells. One hour later the substrate solution (1 mM 3-MeOGal-MU) was added and fluorescent colonies identified and picked. Approximately 100 000 and 12 000 colonies were screened from libraries A and B, respectively, from which 500 and 80 clones, respectively, were chosen. Secondary screening was performed by growing selected colonies in 96-deep well plates, measuring cell density, lysing aliquots and assaying with 3-MeOGal-MU in a 96-well plate reader. No colony with significant activity was found in library B, but two positive clones were identified from library A. The two positive clones identified (Q24S/W404L and Q24N/W404N) each contained two mutations at the same positions and are designated AbgSL and AbgNN.

Kinetic analysis of hydrolytic activity
To compare the hydrolytic activity of the two Abg mutants with that of their parent enzyme, the kinetic parameters of the enzymes were investigated using a range of concentrations of 4-nitrophenyl β-D-glucopyranoside (pNPGl), 4-nitrophenyl β-D-galactopyranoside (pNPGal) and 4-nitrophenyl 3-O-methyl-β-D-galactopyranoside (3-MeOGal-pNP). As expected both mutants cleave pNPGl and pNPGal very slow, with low $k_{cat}$ and high $K_m$ values. However, $k_{cat}/K_m$ values for AbgSL and AbgNN with 3-MeOGal-pNP were 5- and 7-fold higher than those of Abg wild-type, respectively (Table II). The $K_m$ of wild-type Abg for 3-MeOGal-pNP was too high to be determined.
**Generation of a novel Abg glycosynthase and the analysis of its properties**

The mutations obtained through site-directed saturation mutagenesis were introduced into the gene of 2F6 glycosynthase, producing mutants AbgSL-T and AbgNN-T, where the -T suffix indicates a Transferase as opposed to a hydrolase. Gratifyingly these mutants were demonstrated by thin-layer chromatography (TLC) to be active as glycosynthases using 3-O-methyl glucopyranosyl fluoride (3-MeOGlcF) as the donor and pNP-cellobioside as the acceptor (Fig. 3). By contrast, neither the original Abg glycosynthase, AbgE358G (Mackenzie et al., 1998), or its evolved progeny (2F6) had any glycosynthase activity under the same conditions. To ensure that both mutations were important for activity in each case, single mutants were generated separately. These did not produce a glycosynthase reaction product as judged by TLC (data not shown).

**NMR analysis of the reaction product**

To assess the linkage created by the glycosynthase reaction, pNP-cellobioside (12.5 mM) and 3-O-methyl galactopyranosyl fluoride (3-MeOGalF; 60 mM) were combined with AbgNN-T or AbgSL-T (10.2 mg) in 3 ml of Na-phosphate buffer (150 mM, pH 7.5) at 30°C and allowed to stand for 24 h (Fig. 4). TLC analysis indicated >90% yield. After freeze drying and acetylation of the products with acetic anhydride in pyridine, the product was purified by flash column chromatography and analyzed by NMR. Our results indicate that the enzyme catalyzed the formation of β-1,4 linkages, based on the upfield chemical shift (δ 3.80) of H-4 and the downfield chemical shift (δ 5.17) of H-3, affording 3-nitrophenyl 3-O-methyl-β-D-galactopyranosyl-(1,4)-β-D-glucopyranosyl-(1,4)-β-D-glucopyranoside prior to acetylation.

1H NMR (CDCl3, 600 MHz): δ 8.21 (d, 2 H, J 9.2 Hz, Ar-H), 7.06 (d, 2 H, Ar-H), 5.45 (brd, 1 H, H-4', 5.29 (t, 1 H, J2,3 = J3,4 8.6 Hz, H-3), 5.23 (t, 1 H, H-2), 5.19 (d, 1 H, J1,2 7.8 Hz, H-1), 5.17 (t, 1 H, J2',3' = J3',4' 9.3 Hz, H-3'), 4.97 (dd, 1 H, J2',3' 9.7 Hz, H-2''), 4.87 (dd, 1 H, J2',3' 9.3 Hz, H-2''), 4.56 (dd, 1 H, J2',3' 8.2 Hz, H-1'), 4.40 (dd, 1 H, J3',6'a 2.0 Hz, H-6'a), 4.38 (d, 1 H, J1',2' 8.0 Hz, H-1''), 4.20 (dd, 1H, J5',6'b 5.0 Hz, J6'b,6'a 12.0 Hz, H-6'b), 4.14 (dd, 1 H, J5,6a 5.5 Hz, J6a,6b 12.1 Hz, H-6'a), 4.11 (d, 2 H, H-6b & H-6'b), 3.90 (t, 1 H, J3,4 = J4,5 9.2 Hz, H-4), 3.86 (m, 1 H, H-5), 3.80 (t, 1 H, J4,5 = J5,6a 9.5 Hz, H-4'), 3.79 (m, 1 H, H-5'), 3.64 (m, 1 H, H-5), 3.36 (s, 3 H, COCH3), 3.28 (dd, 1 H, J3,4 = J4,5 3.3 Hz, H-3'), 2.16 (s, 3 H, COCH3), 2.15 (s, 3 H, COCH3), 2.11 (s, 3 H, COCH3), 2.10 (s, 3 H, COCH3), 2.09 (s, 3 H, COCH3), 2.07 (s, 3 H, COCH3), 2.06 (s, 3 H, COCH3), 2.04 (s, 3 H, COCH3) and 2.03 (s, 3 H, COCH3).

Electrospray ionization-mass spectrometry: Calcd for [C43H55NO27 + K]': 1056.3. Found m/z: 1056.9.

**Kinetic study of transglycosylation activity**

Kinetic analyses were performed on three enzymes (2F6, AbgSL-T and AbgNN-T) with 3-MeOGalF or 3-MeOGlCF as donor and pNP-cellobioside as acceptor to evaluate the catalysis of β-1,4 linkages. The enzyme catalyzed the formation of β-1,4 linkages, based on the upfield chemical shift (δ 3.80) of H-4 and the downfield chemical shift (δ 5.17) of H-3, affording 3-nitrophenyl 3-O-methyl-β-D-galactopyranosyl-(1,4)-β-D-glucopyranosyl-(1,4)-β-D-glucopyranoside prior to acetylation.

**Fig. 3.** Analysis of the transglycosylation activity of Abg glycosynthase and its mutants by TLC. pNP-cellobioside (10 mM) and 3-MeOGlcF (80 mM) were incubated with the enzymes (3.5 mg/ml) for 24 h; the eluting solvent was 7:2:1 ethyl acetate/methanol/H2O. pNP sugars were detected based on the UV absorbance (365 nm). After 24 h, almost all acceptor molecules (pNP-cellobioside) were utilized in the AbgNN-T reaction.

**Fig. 4.** Synthetic reaction performed by AbgNN-T.
the donor and pNP-cellobioside as the acceptor. In the cases of 2F6 and AbgSL-T, only very low enzymatic activity could be detected (Table III and Fig. 5), and it was impossible to achieve donor saturation. Therefore, their $k_{cat}/K_m$ values were calculated using the slope of the graph of rate versus donor sugar (glycosyl fluoride) concentration. AbgNN-T proved to be the best mutant, with $K_m$ values sufficiently low to observe saturation kinetics, allowing measurement of $k_{cat}$ and $K_m$ values as shown in Table III. A comparison across all three enzymes is possible by considering the reaction using 3-MeOGlcF as donor at a fixed acceptor (pNP-cellobioside) concentration where the $k_{cat}/K_m$ values for AbgSL-T and AbgNN-T were improved by 5- and 39-fold, respectively, relative to 2F6 (Table III and Fig. 5B). To provide insight into acceptor specificity, the kinetic parameters of AbgNN-T were measured with a fixed (110 mM) 3-MeOGlcF concentration at various pNP-cellobioside concentrations. The $k_{cat}$ and $K_m$ values so determined were $0.53 \pm 0.01 \text{ min}^{-1}$ and $7.9 \pm 1.9 \text{ mM}$, respectively. For 3-MeOGalF under the same conditions, the $k_{cat}$ and $K_m$ were $0.14 \pm 0.007 \text{ min}^{-1}$ and $16.7 \pm 1.6 \text{ mM}$, respectively.

**Protein modeling**

Homology-based models of the structures of Abg, AbgSL-T and AbgNN-T were obtained using SWISS-MODEL at the ExPASy server. The position of the glucosyl fluoride shown in this model is based on the position of the 2-fluorosugar in the structure of a trapped 2-deoxy-2-fluoroglucosyl enzyme. In these mutants, two residues (Q24/W404) are substituted by smaller residues (Q24S/W404L or Q24N/W404N; Figs 2 and 6), making more space for the slightly more bulky modified substrates. Mutations at W404 were slightly farther from the C3-OH group; therefore, W404L and W404N likely influenced other C3–OH group-related residues indirectly.

**Discussion**

Rebuilding a previously evolved glycosidase active site to efficiently accommodate an enlarged substrate is a challenging task. However, a combination of saturation mutagenesis at key primary interaction sites around the hydroxyl group of interest with a fluorogenic agar plate-based screen that is capable of processing 100 000 mutants indeed yielded improved mutants. As has been seen with other plate-based assays, a substantial false-positive rate was observed, largely due to asymmetric growth of colonies. However, via use of a relatively simple secondary screen in 96-deep well plates, these were quickly parsed down to the two true hits in which positions Q24 and W404 were modified, AbgSL and AbgNN. As might be expected, these mutations generally replace the original side chain with a less bulky one, to accommodate the additional methyl group.

Lifting these mutations into the optimized 2F6 gene resulted in glycosynthases AbgSL-T and AbgNN-T that are capable of transferring 3-O-methyl-modified glycosyl fluorides to a 4-nitrophenyl cellobioside acceptor. As is the case for reaction of 2F6 glycosynthase with galactosyl fluoride, reaction of 3-O-methyl galactosyl fluoride with these mutants

### Table III. Comparison of the synthetic kinetic parameters of 2F6 and its glycosynthese mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Donor</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (min$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2F6</td>
<td>3-MeOGalF</td>
<td>-$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3-MeOGlcF</td>
<td>ND</td>
<td>ND</td>
<td>0.2</td>
</tr>
<tr>
<td>AbgSL-T</td>
<td>3-MeOGalF</td>
<td>ND</td>
<td>ND</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>3-MeOGlcF</td>
<td>ND</td>
<td>ND</td>
<td>0.94</td>
</tr>
<tr>
<td>AbgNN-T</td>
<td>3-MeOGalF</td>
<td>0.83</td>
<td>277</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>3-MeOGlcF</td>
<td>0.56</td>
<td>72</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Values of $k_{cat}$ and $K_m$ of $0.53 \pm 0.01 \text{ min}^{-1}$ and $7.9 \pm 1.9 \text{ mM}$, respectively, were determined for pNP-cellobioside at a fixed (110 mM) 3-MeOGlcF concentration. Likewise, values of $k_{cat}$ and $K_m$ of $0.14 \pm 0.007 \text{ min}^{-1}$ and $16.7 \pm 1.6 \text{ mM}$ were determined for pNP-cellobioside at fixed (110 mM) 3-MeOGalF.

$^a$The acceptor [pNP-cellobioside] was fixed at 19.5 mM; the error range is from 5 to 10%.

$^b$Not detected.

ND, not determined.

![Fig. 5](image-url) Comparison of the catalytic efficiencies of the parent glycosidase and glycosynthese with mutants. (A) Relative $k_{cat}/K_m$ values for Abg (3-MeGal-pNP, hydrolysis) and mutants. (B) Relative $k_{cat}/K_m$ values for glycosynthese 2F6 and mutants (3-MeOGlcF; transglycosylation).
was limited to a single transfer as the axial configuration of the hydroxyl at C-4 in the product is poorly positioned to function as a nucleophile. However, whereas glucosyl fluoride is transferred multiple times onto the acceptor by 2F6, only a single 3-O-methyl glucosyl moiety is transferred by these mutants, indicating that, perhaps unsurprisingly, 3-O-methyl glucose derivatives do not fit well in the +1 subsite of this enzyme. NMR analysis of the product of transfer of 3-O-methyl galactose onto pNP-cellobioside confirmed the formation of a β-1,4-linkage, thus these modifications had not altered the regiospecificity of the enzyme.

The origins of the measured rate accelerations, and particularly of the 39-fold rate increase (relative to 2F6) seen for AbgNN-T with 3-O-methyl glucosyl fluoride donor are of particular interest. As noted before, the substitutions create more space to accommodate the additional methyl group. This may be further augmented in the case of the glycosynthase mutant by the additional space created through the replacement of Glu358 by glycine. A plausible additional explanation for rate increases centers around Tyr298 in Abg. This was suggested, on the basis of the labeling studies prior to the availability of structures for GH1 glycosidases (Gebler et al., 1995), and through subsequent crystallographic studies with homologues (Aguilar et al., 1997; Sanz-Aparicio et al., 1998; Hakulinen et al., 2000) to play a role in hydrogen bonding with the catalytic nucleophile, Glu358, and with the endocyclic oxygen of the sugar in the -1 site (Fig. 6A).

Creation of the glycosynthase by mutation of Glu358 to Gly removes one of its H-bonding partners, likely compromising its ability to stabilize the glycosynthase transition state. Modeling studies on the AbgNN-T mutant suggest that the introduced N404 restores the hydrogen bonding to Tyr298, thereby stabilizing its interactions with the ring oxygen at the transition state (Fig. 6B). Modeling also suggests that the mutations W404L and W404N also produce a small change in the loop between the eighth β-strand and the eighth helix, leading to the repositioning of Trp412 to better accommodate bulky substrates.

The best glycosynthase mutant thereby obtained, AbgNN-T, is capable of preparative scale (multi-milligram) synthesis of oligosaccharides containing 3-O-methyl glucosides and galactosides wherein only a single sugar residue has been transferred. As such it could be valuable for the preparation of modified glycoconjugates found in natural systems or artificially methyl-capped glycans. Currently, no GTs are known that are capable of such a transfer as methylation is typically a post-glycosylation event. Further, the methyltransferases are also not well known, and would have to be cloned for each instance, whereas a single modified glycosynthase might be applicable to a range of circumstances and use a single methylated glycosyl donor.

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