Directed Evolution of a Glycosynthase from Agrobacterium sp. Increases Its Catalytic Activity Dramatically and Expands Its Substrate Repertoire*

The Agrobacterium sp. β-glucosidase (Abg) is a retaining β-glucosidase and its nucleophile mutants, termed Abg glycosynthases, catalyze the formation of glycosidic bonds using α-glycosyl fluorides as donor sugars and various aryl glycosides as acceptor sugars. Two rounds of random mutagenesis were performed on the best glycosynthase to date (AbgE358G), and transformants were screened using an on-plate endocellulase coupled assay. Two highly active mutants were obtained, 1D12 (A19T, H9251) and 2F6 (A19T, E358G, H9252, M407V, Q248R, M407V) in the first and second rounds, respectively. Relative catalytic efficiencies ($k_{cat}/K_m$) of 1:7:27 were determined for AbgE358G, 1D12, and 2F6, respectively, using α-D-galactopyranosyl fluoride and 4-nitrophenyl β-D-glucopyranoside as substrates. The 2F6 mutant is not only more efficient but also has an expanded repertoire of acceptable substrates. Analysis of a homology model structure of 2F6 indicated that the A19T and M407V mutations do not interact directly with substrates but exert their effects by changing the conformation of the active site. Much of the improvement associated with the A19T mutation seems to be caused by favorable interactions with the equatorial C2-hydroxyl group of the substrate. The alteration of torsional angles of Glu-411, Trp-412, and Trp-404, which are critical for the hydroxyl group of the substrate, are components of the aglycone (+1) subsite, is an expected consequence of the A19T and M407V mutations based on the homology model structure of 2F6.

In recent years, the glycosynthase approach developed in this laboratory has added a new dimension to the enzymatic preparation of oligosaccharides (7–9). Glycosynthases are retaining glycosidase mutants in which the catalytic nucleophile has been converted to a non-nucleophilic residue. These mutants catalyze the formation of glycosidic bonds when glycosyl fluorides with anomeric configuration opposite to that of the normal substrate, thereby mimicking the glycosyl enzyme intermediate, are employed as substrates. The modified enzyme catalyzes the nucleophilic displacement of the fluoride via attack by a hydroxyl group on an added glycosyl acceptor, generating a new glycosidic bond with the same stereochemistry as the normal substrate. The reactions catalyzed by glycosynthases are highly amenable to industrial syntheses because of the high yields of products (70–95%), the relatively inexpensive and easily prepared substrates, and the high stabilities of the enzymes involved (9). Since the first report on a glycosynthase in 1998 (10), successful glycosynthases have been developed from 11 glycosidases belonging to seven different glycoside hydrolases (GH) families (9). With the exception of a unique α-glycosynthase from Schizosaccharomyces pombe (11) all glycosynthases have originated from retaining β-glycosidases, and employ α-glycosyl fluorides as donors. Recently, β-glycosynthases were shown to use activated β-glycosides as donors at low pH in the presence of external nucleophiles, such as acetate or formate (12, 13). This rescue of activity with external nucleophiles permits transglycosylation reactions to be performed via the formation of an intermediate α-glycosyl acetate or formate.

The glycosynthase from Agrobacterium sp. β-glucosidase (Abg) was the first glycosynthase to be reported (10), and its utility has been extended to numerous applications (10, 14–17). The first improvement of Abg glycosynthase activity was achieved by replacing alanine with serine at the nucleophile position2 (18). Subsequently, using an “on-plate” screening method based on a coupled enzyme assay with an endocellu-

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Oligosaccharides have considerable potential as therapeutics because of the numerous medicinally relevant physiological events that involve glycoconjugates (1–3). To expand our understanding of the various roles of oligosaccharides found in important cellular events, more efficient and selective synthetic protocols must be developed for the preparation of oligosaccharides. Classical chemical synthesis is often impractical for the synthesis of complex oligosaccharides because of the need for selective and labor-intensive protection-deprotection steps and difficulties in directing product stereochemistry. To overcome these limitations, enzymatic syntheses using glycosidases or glycosyl transferases have rapidly gained prominence (4–6).

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1 The abbreviations used are: GH, glycoside hydrolases; Abg, Agrobacterium sp. β-glucosidase; Cel5A, β-1, 4-glucanase from C. fimi; CelB, P. furiosus β-glucosidase; Tagg-Gly, T. aggregans β-glycosidase; α-GalF, α-D-galactopyranosyl fluoride; α-GlcF, α-D-glucopyranosyl fluoride; α-ManF, α-D-mannopyranosyl fluoride; α-XylF, α-D-xylopyranosyl fluoride; MU-Glc, 4-methylumbelliferyl β-D-glucopyranoside; pNP-Gal, 4-nitrophenyl β-D-galactopyranoside; pNP-Glc, 4-nitrophenyl β-D-glucopyranoside; pNP-Man, 4-nitrophenyl β-D-mannopyranoside; pNP-Xyl, 4-nitrophenyl β-D-xylopyranoside.

2 The amino acid sequence numbering system used here is that which has been used throughout for this protein in which the N-terminal methionine (which is processed off in the mature protein) is not counted. This results in a numbering difference of 1 compared to that listed in GenBank™ (accession number M19033), thus the nucleophile of Abg is referred to as the 358th amino acid residue rather than the 359th (52).
lase, an improved new glycosynthase, AbgE358G with Gyl at the nucleophile position, was discovered from a library of mutations at the catalytic nucleophile position (19). However, the reactions catalyzed by these improved Abg glycosynthases are still slow relative to wild type glycosidase activities, requiring relatively large quantities of mutant enzyme and/or extended incubation times (9, 17, 18). This has stimulated attempts to generate more active glycosynthase mutants.

In this report, we present a modified screening method and its use on a mutated gene library to identify a mutant enzyme, designated 2F6, containing three additional mutations and a 27-fold enhancement in catalytic efficiency relative to the parental glycosynthase (AbgE358G) after two rounds of mutagenesis and screening. The enhanced activity also allows 2F6 to use other sugars as donors and acceptors. We also propose a structural basis for these activity changes based upon a suitable homology model.

EXPERIMENTAL PROCEDURES

Construction of pGSVIII as a Screening Vector—The gene encoding the catalytic domain of cellulase D (Cel5A) from Cellulomonas fimi (20) was amplified by PCR using 1 μM T7 promoter primer (5'-TAATACGACACTGTAGG-3') and the Cel5A-TERM primer (5'-GCCTCAGATTTATAGCTGACCTGAATTTTAAGCTTGACCTGGAGATCA-3') each 0.2 μM of each of the four dNTPs, 5% dimethyl sulfoxide, 25 ng of pGVIICel5A (19), template DNA, and 2.5 units of Pco polymerase (Roche Diagnostics) in 50 μM of 1× Pco polymerase buffer. Twenty-five PCR cycles (45 s at 94°, 30 s at 55°, and 90 s at 72°) were performed in a thermal cycler (PerkinElmer Life Sciences, GeneAmp PCR System 2400). The resulting PCR product was digested with T7 promoter and A19 reverse and then cloned into pTKN1119, which carried the Bacillus licheniformis maltohydrolase amylase promoter (21), multiple cloning sites, the hexahistidine tag sequence, T7 terminator, and the kanamycin resistance gene from pET29 bait (+) (Novagen). The resulting plasmid was designated as pGSVIII and was used for the dual expression of the catalytic domain of Cel5A and His-tagged Abg glycosynthase mutants in this study.

Random Mutagenesis and Construction of Mutant Library—Random mutagenesis of the template gene was performed using unbalanced dNTP concentrations in the presence of MnCl2 (22). The glycosynthase gene was amplified from pET29abgE358G with the T7 promoter primer and the Abg-TERM primer (5'-GGCGCTCGAGTGGGCGCCCGCTTGGGCGACCCCATGGT) using 2 μM of each primer, 5 μM dNTPs, 50 ng of DNA, 50 μM dCTP, 50 μM dGTP, 25 μM of pGVIICel5A (19), 10 units of Pco polymerase in 100 μL reaction volume. Cycling parameters were 35 cycles of 95° for 1 min, 55° for 1 min, and 72° for 2 min. The PCR products were digested with XbaI and NotI, then extracted from agarose gel using a QuIAgk Gel Extraction Kit (Qiagen) and ligated with pGSVIII that had been digested with XbaI and NotI and then transformed into DH5α competent Escherichia coli cells. Transformants were isolated on Luria-Bertani LB (50 μg/mL kanamycin) plates, and the expression of the catalytic domain of Cel5A and His-tagged Abg glycosynthase mutants in this study were confirmed using the same conditions as those used for the secondary screening.

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Ordinary glycosynthase enzymes are relatively large quantities of mutant enzyme and/or extended incubation times, requiring extensions from an active clone were monitored under a UV light (395 nm) and the brightest clones (~10 clones per 300 colonies) were then picked from master plates and transferred into 96-deep well culture tubes, each well containing 200 μL of LB medium. The primary screened clones were then incubated overnight at 37° C with shaking at 250 r.p.m. to allow for cell growth. Seventy-five microliters of each culture were transferred to new 96-well plates and the rest of the cultures were stored at 4° C. Bacterial growth was monitored by measuring the absorbance at 600 nm using a spectrophotometer (SPECTRAMax plus, Molecular Devices Corp.). An equal volume (75 μL) of BugBuster™ protein extraction reagent (Novagen) with 0.1% (w/v) lysozyme (Sigma) was then added to each well and the plates were incubated for 30 min at room temperature to lyse the cells. Next, the crude extracts were mixed with an equal volume of assay solution (25 μM α-GlcF, 2 mM MU-Glc, 0.1 mg/mL Cel5A in 100 mM potassium P, pH 7.0, and the release of methylumbelliferone (MU) was monitored at 30° C using a Wallac 1420 VICTOR2 (PerkinElmer Life Sciences) plate reader. Clones with higher activity than that of the parent clone in the secondary screening were purified and their improved activities were confirmed using the same conditions as those used for the secondary screening.

Purification of Glycosynthase Mutant Enzymes—The Abg glycosynthase mutants were purified from E. coli TOP10 cells harboring the corresponding genes on pGSVIII by affinity chromatography using nickel-nitrotriacetate agarose (Qiagen), as described previously (18). The desalting and the concentration of purified enzyme solutions were carried out using an Amicon Ultra-4 filter unit (10,000 Da, cut-off, Millipore). The buffer used in enzyme solutions was changed to 100 mM potassium phosphate buffer (pH 7.0). Protein concentrations were determined by measuring absorbance at 280 nm, using an extinction coefficient of 102850 M⁻¹ cm⁻¹ (23).

Transglycosylation Kinetics of Glycosynthase—An Orion fluoride electrode monitor model 96–088N (Orion Research, Inc.) was interfaced with a Fisher Scientific Accumet 925 pH/ion meter, was used to monitor fluoride release during reaction at 25° C. All enzymatic rates were recorded for the spontaneous hydrolysis rate of the glycosyl fluoride. The concentration of either donor (50 mM) or acceptor (20 mM) sugar was fixed and that of the acceptor was varied to allow Kcat and kcat determinations. The apparent kcat/Km value of 2F6 for pNP-Xyl was determined from the slope of the plot of v versus [S] (V = kcat [S]/Km) at eight concentrations of pNP-Xyl from 1 to 20 mM at a fixed concentration of α-GalF (50 mM). GraFit version 4.0 (24) was used to calculate kinetic parameters by direct fit of initial rates.

Oligonucleotide Synthesis and DNA Sequencing—The synthesis of PCR primers and the analysis of DNA sequences were carried out by the Nucleic Acid and Peptides Service Unit in the Biotechnology Laboratory at the University of British Columbia.

Structure Modeling—The three-dimensional structures of Abg and the glycosynthase mutants were modeled using the SWISS-MODEL version 3.51 program at the ExPasy server (25). Representations of 2F6 and the other structures were created by using Raster3D version 2.1 (32) and rendered using Raster3D (33).

RESULTS

Modification of the Screening System—To improve the efficiency of formation of screenable mutant libraries, the previ-
ously developed two-plasmid screening method (19) was slightly modified to minimize some earlier limitations. Previously, it had been necessary to use *E. coli* BL21(DE3) as a host cell and to employ isopropyl β-D-thiogalactopyranoside induction because the expression of the target gene was under T7 promoter control. Furthermore, the two plasmids for Cel5A and the glycosynthase had to both be transformed to produce these enzymes in cells simultaneously, and because Cel5A also contained a His$_6$ tag, the two plasmids then had to be separated to purify the improved glycosynthase after screening. To overcome these limitations, the T7 promoter was changed to a constitutive *B. licheniformis* maltogenic amylase promoter (21), and a single plasmid system was designed. In the single plasmid system, the gene for Cel5A was manipulated to produce Cel5A without a His$_6$ tag followed by a second ribosome binding site, which originated from pET29 (Novagen), along with a His$_4$-tagged glycosynthase mutant gene. Conveniently, both genes are transformed through a single transformation process and also expressed in any *E. coli* host cell without isopropyl β-D-thiogalactopyranoside induction. Glycosynthase mutants produced by the screened clones can be directly purified using nickel affinity chromatography.

Random Mutagenesis and Screening—Error-prone PCR mutagenesis using MnCl$_2$ and unbalanced dNTP concentrations was used to generate libraries of glycosynthase mutants (22). In the first generation, to optimize the concentration of MnCl$_2$, the mutant genes were amplified at various MnCl$_2$ concentrations ranging from 0.1 to 1 mM using the *abgE358G* gene as a template. About 90% of the clones in a mutant library made in the presence of 0.5 mM MnCl$_2$ exhibited positive glycosynthase activity in the on-agar plate assay, and sequence analysis of 5 randomly selected clones in the library showed a nucleotide mutation ratio of −0.06%, which is an ideal mutagenesis ratio for directed evolution (one amino acid substitution per gene). After screening 10,000 colonies from the mutant library, only one mutant (1D12) was selected. Sequence analysis of 1D12 revealed one amino acid substitution, A19T. This substitution resulted in 3.5 times higher activity than that of AbgE358G in the screening assay (see “Experimental Procedures”). The gene encoding 1D12 was then used as a template for generating the second generation error-prone PCR library under the same conditions (0.5 mM MnCl$_2$) as the first generation. An additional improved mutant, 2F6, showing 1.5 times more activity than 1D12, was revealed after screening 10,000 colonies of the second library. The new mutant had acquired three mutations compared with the AbgE358G: it retained both amino acid substitutions of its parent, A19T and E358G, and acquired two additional mutations, Q248R and M407V. To allow analysis of individual mutations and see if any of these were silent, two new mutants, 2F61 (A19T, E358G, and Q248R) and 2F62 (A19T, E358G, and M407V), were prepared by recombination using restriction enzymes. In fact, both of these mutants were more active than 1D12 (1.1 and 1.2 times for 2F61 and 2F62, respectively) thus all three mutations acquired in the directed evolution process do indeed contribute to the increased glycosynthase activity.

In an attempt to further improve activity, the three mutated sites were randomized individually by saturation mutagenesis (34) using the *abgE358G* gene as a template. The screening of ~900 colonies (~300 colonies from each of the three saturation mutagenesis libraries) revealed no new mutation that satisfied the selection criteria. Only one mutant, A19X-40, which contained an A19S conversion, was identified as an improved mutant (2.5 times higher than AbgE358G), but its activity was less than that of 1D12 having the A19T mutation (Fig. 1).

**Improvement of Glycosynthase Activities**—To compare the improved transglycosylation activity of two screened AbgE358G mutants, we investigated the kinetic parameters of these mutants with α-D-galactopyranosyl fluoride (α-GalF) as a donor and 4-nitrophenyl β-D-glucopyranoside (pNP-Glc) as an acceptor. The use of α-GalF as a donor sugar for kinetic studies ensured that the observed release of fluoride corresponded to a single galactosyl transfer because the lactosyl product, with an axial 4′ hydroxyl, does not act as an acceptor. Upon varying the α-GalF concentration at a fixed concentration of pNP-Glc (20 mM) all three glycosynthases showed standard saturation kinetic behavior (Fig. 2). The catalytic turnover numbers ($k_{cat}$) of two screened mutants were higher by 10- and 15-fold than that of AbgE358G (Table I). The $K_m$ value for α-GalF increased with the A19T mutation from 316 to 422 mM but decreased with the two additional mutations (Q248R and M407V) to 174 mM. Overall, the $k_{cat}/K_m$ of Abg glycosynthase was improved 27-fold relative to that of AbgE358G through this directed evolution process.

**Acceptor Specificity**—To investigate the effect of the triple mutations on the acceptor specificity of the glycosynthase, kinetic parameters were determined for various acceptor sugars that have an equatorial hydroxyl group at the 4-position, using a fixed donor sugar concentration (α-GalF, 50 mM). The values of $k_{cat}$ for all acceptor sugars increased more than 10 times relative to that of AbgE358G (Table II). In the case of pNP-Glc, both AbgE358G and 2F6 suffered substrate inhibition, but 2F6 was inhibited less than AbgE358G (the values of $K_i$ for AbgE358G and 2F6 were 7 and 24 mM, respectively). In the case of 4-nitrophenyl β-D-mannopyranoside (pNP-Man), standard saturation kinetic behavior was observed for AbgE358G with the $k_{cat}$ for 2F6 being 10 times higher than that of AbgE358G. $K_m$ values for pNP-Glc and pNP-Man did not change significantly. By contrast, $K_m$ values for 2F6 with 4-nitrophenyl β-D-xlyopyranoside (pNP-Xyl) dramatically increased, so saturation kinetics were not observed, despite the $k_{cat}/K_m$ value increasing from 5.5 to 74 min$^{-1}$ mM$^{-1}$.

**Donor Specificity**—To investigate glycosynthase activities for new donor substrates, α-D-xlyopyranosyl fluoride (α-XylF) and α-D-mannopyranosyl fluoride (α-ManF) were tested (Table III). AbgE358G showed little activity for the glycosylation of pNP-Glc with α-XylF ($V_0 \sim 0.1$ min$^{-1}$ with 80 mM α-XylF and 20 mM pNP-Glc). By contrast a value of 3.4 min$^{-1}$ was determined for 2F6 under the same conditions, which translates to a 34-fold improvement. Saturation was not observed until an

**FIG. 1.** Directed evolution of Abg glycosynthase for activity improvement. Activities are measured by use of the coupled assay to monitor the release of MU and represent a relative value based on that of AbgE358G. The names of the Abg glycosynthase variants and newly generated amino acid substitutions are labeled on the corresponding bar and arrow, respectively.
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Fig. 2. Comparisons of transglycosylation kinetics with AbgE358G, 1D12, and 2F6. Reaction rates of AbgE358G (○), 1D12 (■), and 2F6 (□) were measured using a fluoride electrode. pNP-Glc is used as an acceptor fixed at 20 mM. All assays were performed at 25°C in 100 mM potassium Pi (pH 7.0) containing 150 mM NaCl. The error range for data in this figure is from 5 to 10%.

α-XyIF concentration of 400 mM yielding values of \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) of 53 min\(^{-1}\) and 0.05 mM\(^{-1}\) min\(^{-1}\), respectively. In the case of α-ManF, with an axial C2-hydroxyl group, the \( k_{\text{cat}} \) of 2F6 (39 min\(^{-1}\)) increased by only 2-fold relative to AbgE358G (18 min\(^{-1}\)). The catalytic efficiency (\( k_{\text{cat}}/K_m \)) for α-ManF increased by 6-fold because of the increased affinity of 2F6 for α-ManF (\( K_m \) values for AbgE358G and 2F6 were 150 and 68 mM, respectively), a relatively small improvement compared with α-glycosyl fluorides with an equatorial C2-hydroxyl group.

Protein Modeling—To investigate why the observed mutations enhanced the activity, homology-based model structures of AbgE358G and 2F6 were obtained using the Swiss protein modeling server (25). Based on the model structures, all of the three mutations are too far from the substrate to participate in direct interactions; the distance between the anomic center of the substrate and Co of A19T and M407V being 9 and 13 Å, respectively, whereas Q248R is found on the surface of Abg (Fig. 3). Therefore, it is likely that the mutations exert their effects by changing the conformation of the active site rather than by interacting directly with substrates.

DISCUSSION

Directed evolution is one of the most powerful tools presently available to improve the characteristics of enzymes (35–37). The success of the directed evolution approach depends on the efficient formation of a random mutant library and a highly quantitative and high-throughput screening system. In the reported previous study (19), we developed an on-agar plate-coupled enzyme assay based on a two-plasmid system allowing the selection of active glycosynthases from a mutant library. In this study, we modified the two-plasmid system to a single plasmid system to improve efficiency. Additionally, to reduce the requirement for very high donor substrate concentration (1 M), a cell wall lysis step involving β-cycloserine, a mild cell lysis reagent, was added (38). Consequently, α-GlcF easily permeated through the damaged cell wall, and the MU oligosaccharides formed through the glycosynthase activity were not dispersed but accumulated in cells, allowing for facile screening of active clones. However, whereas extremely useful as a fast screen this plate assay was still not quantitative, perhaps because of the unequal growth of cells on agar plates and the inefficient expression of Cel5A (≤0.5 mg/liter culture) compared with the glycosynthase (≤50 mg/liter culture) in the present expression system. The reason for such an unbalanced expression is unclear but low-level expression of cellulases from C. fimi in E. coli systems has previously been reported (20, 39). To address these problems, a 96-well plate assay was designed for positive clones that had been regrown. Unequal cell growth was corrected for by standardization to total cell growth by division of the rate observed by the absorbance at 600 nm. Addition of extra coupling enzyme (Cel5A) to each well ensured that this latter step was not rate-limiting.

Through two rounds of random mutagenesis and screening, we have identified a catalytically improved Abg glycosynthase mutant (2F6) with a significantly higher \( k_{\text{cat}}/K_m \) and that contained only three amino acid substitutions (Fig. 1, Table I). These mutations led to improved Abg glycosynthase activity for a range of donor and acceptor sugars (Tables II and III). Comparison of the rates observed for each donor suggests a rationale for the observed rate enhancements. Wild type Abg has high \( k_{\text{cat}} \) values for pNP-Glc, 4-nitrophenyl β-o-galactopyranoside (pNP-Gal), and 4-nitrophenyl β-o-fucopyranoside (pNP-Fuc) but low values for pNP-Man and pNP-Xyl (40). These results are consistent with the detailed analysis of Namchuk and Withers (41) and suggested that the transition state of Abg is stabilized much more effectively by interactions with the equatorial C2-hydroxyl group compared with the C4- and C6-hydroxyl groups. Indeed the C2-hydroxyl group was shown to contribute at least 18 and 22 kJ/mol to transition state stabilization for glycosylation and deglycosylation, respectively (41). In addition, removal of the entire C6-hydroxymethyl group greatly increased the activation energy. In this study, the greatest improvements observed upon evolution to 2F6 are for those substrates containing an equatorial C2-hydroxyl group, such as α-GalF, α-GlcF, and α-XyIF (Table III). By contrast the improvement for α-ManF was much smaller. Therefore, the structural changes effected by the mutations to create 2F6 primarily resulted in greater transition state stabilization through improved interactions with the equatorial C2-hydroxyl group of the substrate.

In the absence of a three-dimensional structure, the model structure generated using known structures of homologous enzymes is helpful in understanding the roles of the various mutations in improving the characteristics of enzymes through directed evolution. We therefore tried to dissect out the possible consequences of the three individual mutations in 2F6 using the homology-modeled structure along with the known structures of β-glucosidases from GH family 1. As is graphically illustrated in Fig. 1, the substitutions of Ala-19 in AbgE358G to Ser or Thr both improved activity, with the Thr substitution giving the most enhancement (Fig. 1). We therefore believe that the hydroxyl group of Thr-19 in 1D12 and Ser-19 in A19X-40 improve glycosynthase activity through similar mechanisms, with the methyl group of Thr-19 in 1D12 providing a further enhancement of activity, presumably by inducing a better alignment of active site residues. Among the reported structures of β-glucosidases the enzyme from Bacillus circulans (Protein Data Bank code 1QOX (28)) has an alanine (Ala-15) at this position, whereas the Thermosphaera aggregans enzyme (TAβ-Gly, Protein Data Bank code 1QVB (30)) has a serine (Ser-13) at this position. Inspection of superposed structures of 1QOX and 1QVB reveals that the backbone of 1QVB is slightly distorted, and Ser-12 in 1QVB forms a hydrogen bond with the backbone oxygen atom of Thr-385 (Fig. 4A). The substitution of Ser-12 in Taβ-Gly to Thr, as observed in 2F6, would allow the hydroxyl side chain of S12T to form H-bonds with the backbone oxygen atom of Thr-385 and NH1 of Arg-78 in the vicinity of Ser-12. Arg-78 interacts with Asn-207 and the catalytic nucleophile (Glu-386) in 1QVB via three hydrogen bonds and seems to play an important role in maintaining the active site structure. As is known from the mutagenic analysis of the β-glucosidase from Pyrococcus furiosus (CelB) (42), Arg-78 (Arg-77 in CelB) as well as Asn-207 (Asn-206 in CelB) in 1QVB participate in the ground state binding of substrates with an
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Michaelis-Menten parameters were measured with respect to α-GalF donor, with pNP-Glc fixed at 20 mM at pH 7.0, 25°C using a fluoride electrode. Error range is from 5 to 10%.

| Variant     | Amino acid substitutions | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $k_{cat}/K_m$ |
|-------------|--------------------------|-----------|-------|---------------|---------------|
|             |                          | min⁻¹     | mM    | min⁻¹ mM⁻¹    |               |
| AbgE358C   | E358C                    | ND        | ND    | 0.018         |               |
| AbgE358A   | E358A                    | ND        | ND    | 0.034         |               |
| AbgE358S   | E358S                    | 177       | 220   | 0.80          |               |
| AbgE358G*  | E358G                    | 210       | 118   | 1.8           |               |
| AbgE358G   | E358G                    | 551       | 316   | 1.7           |               |
| 1D12       | + A19T                   | 4958      | 422   | 11.7          |               |
| 2F6        | + Q248R, M407V           | 8282      | 174   | 47.3          |               |

* Data from Ref. 19.
*ND, not determined.
* Data from Ref. 18.

TABLE II

Comparison of the kinetic parameters of AbgE358G and 2F6 for various acceptors with α-GalF fixed at 50 mM

| Mutant | Acceptor | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | Behavior (at high substrate concentration) |
|--------|----------|-----------|-------|---------------|-------------------------------------------|
|        |          | min⁻¹     | mM    | min⁻¹ mM⁻¹    |                                           |
| AbgE358G | pNP-Glc  | 310 ± 107 | 45 ± 18 | 6.9           | Substrate inhibition $K_i = 7 ± 3$ mM |
|         | pNP-Man  | 25 ± 1    | 22 ± 2 | 1.1           | Saturation                                |
| 2F6     | pNP-Glc  | 50 ± 3    | 9 ± 1  | 5.5           | Saturation                                |
|         | pNP-Man  | 4498 ± 942 | 31 ± 8 | 145.1         | Substrate inhibition $K_i = 24 ± 7$ mM |
|         | pNP-Xyl  | 278 ± 14  | 25 ± 2 | 11.1          | Saturation                                |
|         | pNP-Xyl  | ND        | ND     | 74            | Linear                                    |

*ND, not determined.

TABLE III

Comparison of the kinetic parameters of AbgE358G and 2F6 for various donors and pNP-Glc fixed at 50 mM

| Donor   | Mutant | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $k_{cat}/K_m$ |
|---------|--------|-----------|-------|---------------|---------------|
|         |        | min⁻¹     | mM    | min⁻¹ mM⁻¹    |               |
| α-GalF  | AbgE358G | 551       | 316   | 1.7           |               |
| α-ManF  |              | 18        | 150   | 0.1           |               |
| α-XylF  |              | ND        | ND    | ND            |               |
|         | 2F6     | 8282      | 174   | 47.3          |               |
|         |              | 39        | 68    | 0.6           |               |
|         |              | 53        | 1060  | 0.05          |               |

*ND, not determined.

equatorial C2-hydroxyl group and contribute to transition state stabilization. Such a focus of productive mutations upon improving transition state interactions with the C2-hydroxyl group is perhaps unsurprising because our earlier studies regarding this and other β-glucosidases have shown that interactions at the 2-position can contribute up to 10 kcal/mol to transition state binding (41, 43–45). Furthermore, structural studies on several of these enzymes had revealed that the most important contribution to such interactions was in fact the carbonyl oxygen of the nucleophile (45–47). Creation of the glycosynthase by mutation of Glu-358 thereby not only removes the ability to form a glycoyl-enzyme intermediate, but also removes a key stabilizing interaction to the substrate C2-hydroxyl group. It is therefore probable that repositioning of A19T in 2F6, as in 1QVB, allows formation of new hydrogen bonds between A19T, Arg-81, and Thr-358, and that these conformational changes largely restore the transition state interactions between the enzyme and the equatorial C2-hydroxyl group (Fig. 4B). Consequently, the activity of 2F6 for α-ManF with an axial C2-hydroxyl group improved less than for the substrates with an equatorial C2-hydroxyl group (Table III). It would therefore be interesting to carry out a separate round of directed evolution employing α-ManF as donor and discover what mutations occurred in that case.

The repositioning of residues in the glycone subsite (also known as the (−1) subsite (27, 48)) is an expected consequence of the A19T and M407V conversions (Figs. 4B and 5). The...
substrate subsite of β-glycosidases in GH family 1 contains six highly conserved amino acid residues (Gln-24, His-125, Asn-169, Trp-404, Glu-411, and Trp-412 in Abg). These conserved residues in the glycone pocket play important roles in recognizing substrates (49, 50). Met-407 in AbgE358G is located in the loop between the 8th strand and the 8th helix and Glu-411, Trp-412, and Trp-404, which are components of the glycone (−1) subsite and interact with the equatorial C3- and C4-hydroxyl groups of glucose, are placed at the ends of this loop (Fig. 5). The hydrophobic aliphatic chain of Met is surrounded by five hydrophobic residues: Ile-25, Cys-59, Leu-406, Val-423, and Val-425. This hydrophobic arrangement is commonly found in β-glucosidase structures. Therefore, it seems that Met-407 plays an important role in directing hydrophobic core structure, and substitution with the branched side chain of valine could lead to a significant conformational change in the peptide backbone around M407V. Additionally, the repositioning of A19T next to Trp-404 allows the methyl group of A19T to contact the ring of Trp-404 (Fig. 4B). Consequently, these conformational changes in the region from Trp-404 at the end of the 8th strand to Trp-412 induced by A19T and M407V seem to adapt the active site of 2F6 to a suitable form to act on α-glucosyl fluoride, which is not a substrate for wild type Abg. In the case of the Q248R substitution, the reason for improvement is less clear, as this residue is found on the protein surface (Fig. 3).

Finally, the greater glycosylation activity of 2F6 can be expected not only to improve product yields and to reduce reaction times but also to broaden the synthetic repertoire. A useful example might be in the creation of an enzyme to synthesize xylo-oligosaccharides. Indeed, our group has also tried unsuccessfully to make a xylosynthase from β-xylanases and xylanases in GH families 3, 10, 11, and 39. Whereas a successful enzymatic synthesis of β,1,4-linked pNP-oligosaccharides from disaccharide to heptasaccharide was reported using a wild type β-xylanase from Aspergillus niger (51), the modest yield obtained is insufficient for industrial application. Hence, 2F6 gives us a new approach for development of xylosynthases through directed evolution using 2F6 as a parent enzyme.

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