

Conversion of a Cyclodextrin Glucanotransferase into an α -Amylase: Assessment of Directed Evolution Strategies[†]

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ABSTRACT: Glycoside hydrolase family 13 (GH13) members have evolved to possess various distinct reaction specificities despite the overall structural similarity. In this study we investigated the evolutionary input required to efficiently interchange these specificities and also compared the effectiveness of laboratory evolution techniques applied, *i.e.*, error-prone PCR and saturation mutagenesis. Conversion of our model enzyme, cyclodextrin glucanotransferase (CGTase), into an α -amylase like hydrolytic enzyme by saturation mutagenesis close to the catalytic core yielded a triple mutant (A231V/F260W/F184Q) with the highest hydrolytic rate ever recorded for a CGTase, similar to that of a highly active α -amylase, while cyclodextrin production was virtually abolished. Screening of a much larger, error-prone PCR generated library yielded far less effective mutants. Our results demonstrate that it requires only three mutations to change CGTase reaction specificity into that of another GH13 enzyme. This suggests that GH13 members may have diversified by introduction of a limited number of mutations to the common ancestor, and that interconversion of reaction specificities may prove easier than previously thought.

The glycoside hydrolase family 13 (GH13), part of the α -amylase superfamily clan H (GH13, 70, 77), represents the largest family of glycoside hydrolases acting on starch and related saccharides (1–3). Members of GH13 catalyze cleavage of α -glycosidic bonds between two glucose residues, forming a covalent glycosyl–enzyme intermediate, but vary considerably in reaction specificity (4). It is surprising that a family of structurally similar proteins containing four conserved motifs, a similar catalytic machinery, and an identical double displacement catalytic mechanism displays such varied reaction specificities for its individual members (1, 5). Cyclodextrin glucanotransferases (CGTases¹) primarily catalyze transglycosylation reactions from starch in which the acceptor is the nonreducing end of the covalent glycosyl–enzyme intermediate (6). This nonreducing end sugar is guided into position for nucleophilic attack on the covalent intermediate by residues of the acceptor subsites, resulting in formation of a circular saccharide or cyclodextrin (cyclization) (7). Structurally related α -amylases initially apply an identical sequence of catalytic events, but hydrolyze starch using a water molecule as acceptor. It was therefore of interest to investigate the amino acid regions, or even single residues, responsible for reaction specificity differentiation. For this purpose, we applied directed evolution to CGTase,

a well-studied member of family GH13. In an effort to evolve the cyclization specificity of the *Thermoanaerobacterium thermosulfurigenes* strain EM1 (Tabium) CGTase into α -amylase like hydrolytic activity on starch, we separately applied error-prone PCR (epPCR) and saturated mutagenesis, also evaluating the effectiveness of directed evolution techniques for interconversion of reaction specificities. Directed evolution has emerged as a powerful method for the creation of proteins with altered properties, *e.g.*, tighter binding, resistance to denaturing conditions, and modified and/or improved reaction specificities (8–13). Several directed evolution methods are commonly used nowadays, epPCR, DNA shuffling, site-saturation mutagenesis, etc., in the generation of genetic diversity, as reviewed (14, 15). With the availability of both structural and mechanistic information of CGTases, semirational mutagenesis, allowing design and generation of a combined saturated set of mutant amino acids, was selected as the initial evolutionary approach. Such close proximity of mutations may allow for complementation of each other's contributing effects to the selected catalytic reaction, otherwise unattainable through single substitutions. Combined saturation mutagenesis has been applied successfully to broaden the substrate range of enzymes (16), and for the site specific incorporation of unnatural amino acids into proteins *in vivo* (17). Residues of the acceptor subsites of CGTase were targeted for semirational mutagenesis due to their importance in the final stages of the cyclization reaction. In order to reduce the possibility of misidentification of effective mutations at unpredicted locations elsewhere in the protein, we applied epPCR throughout the entire *cgt* gene. Our results show that it is possible to effectively interchange reaction specificities within GH13, demonstrated by conver-

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¹ Abbreviations: CGTase, cyclodextrin glucanotransferase; G5-pNP, *p*-nitrophenol maltopentaoside; pNP, paranitrophenol; Tabium, *Thermoanaerobacterium thermosulfurigenes* strain EM1; epPCR, error-prone polymerase chain reaction.

sion of a transglycosylase into an efficient hydrolase through combined saturation mutagenesis.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Protein Production. *Escherichia coli* strain MC1061 was used for DNA manipulations and library screening. CGTase proteins were produced and purified using *Bacillus subtilis* strain DB104A carrying plasmid pCScgt-tt, as described (18). Plasmid carrying strains were grown on LB medium at 37 °C in the presence of kanamycin (50 µg/mL for *E. coli* and 5 µg/mL for *B. subtilis*). Purity and molecular weight of CGTase proteins were checked by SDS–PAGE. Enzyme concentrations were determined using the Bradford reagent from Bio-Rad (München, Germany) and bovine serum albumin as standard.

DNA Manipulations. Mutants were constructed in pCScgt-tt as described (19) and verified by DNA sequencing. Mutant F260W was constructed by digesting the A231V/F260W template with the restriction enzymes *Stu*I, *Dra*III (Roche), and cloning the F260W fragment into the wild-type gene.

Saturation Mutagenesis. Phe184, Ala231, Phe260 of Tabium CGTase were replaced by all nineteen other amino acid residues by site-saturation mutagenesis, using the following oligonucleotides: A231X, 5'-ATA CGT CTA GAT NNS GTA AAA CAT ATG-3'; F260X, 5'-TTT GGA GAG TGG NNS CTT GGA ACG AAT-3'; F184X, 5'-GGA ACA GAT NNS TCA TCT TAT-3'. Underlined regions of oligonucleotides indicate where the nucleotide substitution was introduced. N is A + G + C + T, S is G + C, and X is any amino acid residue. The A231X library was constructed using wild-type pCScgt-tt as PCR template. The A231X/F260X library was made using the pCScgt-tt-A231X library as template. Finally, the A231V/F260W/F184X library was generated using pCScgt-tt-A231V/F260W as PCR template.

Error-Prone PCR Mutagenesis. The *cgt* gene was amplified from pCScgt-tt with the primers For1 (*Nco*I), 5'-GGC TTT TCA GCC CTG CCC AGG CCA TGG-3' and Rev1 (*Kpn*I), 5'-GTT TAC AAT TAC GGT ACC TGT ACT AGA-3'. Restriction sites are underlined. PCR mixtures (50 µL) contained: 1x Taq DNA polymerase buffer, 1 mM MgSO₄, 0–0.3 mM MnCl₂, 0.6 mM of each dNTP, 0.07 µM of each primer, 20 ng of template, and 2.5 units of Taq DNA polymerase (Roche). PCR reactions were performed for 25 cycles: 30 s 94 °C, 40 s 54 °C, and 2 min 72 °C. The PCR products were restricted with *Nco*I and *Kpn*I, and the resulting fragment (2100 bp) was extracted from agarose gel (QIAquick Gel Extraction Kit; Qiagen) and cloned in pCScgt-tt, replacing the wild-type *cgt* gene.

Enzyme Assays. All enzyme assays were performed in 10 mM sodium citrate buffer (pH 6.0) at 60 °C. β -Cyclodextrin forming activity was determined by incubating 1.3–66 nM of enzyme with a 2.5% (w/v) solution of partially hydrolyzed potato starch with an average degree of polymerization of 50 (Paselli SA2; AVEBE, Foxhol, The Netherlands). The amount of β -cyclodextrin produced was quantified with phenolphthalein (20). Starch hydrolyzing activity was measured by following the increase in reducing power with dinitrosalicylic acid, using 1% (w/v) soluble starch (Lamers & Pleuger, Belgium) and 13 nM of enzyme (21). For comparison, hydrolytic rates of the *Bacillus licheniformis*

(Sigma) and *Aspergillus niger* (Megazyme, Wicklow, Ireland) α -amylases were also measured using the aforementioned assay. Hydrolysis of the *p*-nitrophenol maltopentaoside, (G5-pNP) (Megazyme, Wicklow Ireland) substrate was measured in a discontinuous assay by following the release of pNP. One hundred microliters of reaction samples was added to 1 mL of 1 M Na₂CO₃ prior to absorbance measurement at 401 nm. Under such assay conditions the molar extinction coefficient of pNP is 18.4 M⁻¹ cm⁻¹ at 401 nm.

Selection of CGTase Variants with Increased Activity. *E. coli* MC1061 transformed with the library was plated on LB agar plates, and the resulting colonies were transferred to 200 µL of LB medium in 96-well microtiter plates using the Q-pix robot (Genetix, New Milton Hampshire, U.K.) followed by incubation overnight (750 rpm) at 37 °C. For starch hydrolysis, 25 µL of each culture was transferred to a second 96-well plate containing 25 µL of bacterial protein extraction reagent (Pierce, Rockford, IL) per well to lyse the cells. Subsequently, 200 µL of 1% (w/v) soluble starch (Lamers and Pleuger, Wijnegem, Belgium) in 10 mM sodium citrate buffer (pH 6.0) was added, and the microtiter plates were incubated at 60 °C for 2 h. The amount of reducing sugars formed was measured using an adapted version of the Nelson–Somogyi assay, as described (22). For the β -cyclization activity screen assay the same parameters were used for cell growth and lysis as for hydrolysis screening selection, followed by incubation of the cell lysate in 200 µL of 1% (w/v) partially hydrolyzed potato starch (Paselli SA2; AVEBE, Foxhol, The Netherlands). Microtiter plates were then incubated at 60 °C for 2 h. The amount of β -cyclodextrin formed was measured by the addition of a 10 µL sample to 100 µL of phenolphthalein solution (20).

HPLC Product Analysis. Formation of cyclodextrins and linear products from 10% (w/w) starch (Paselli SA2; AVEBE) was analyzed by incubating the starch solution for 24 h with 65 nM of wild-type and mutant proteins (A231V/F260W, A231V/F260W/F184H, and A231V/F260W/F184Q). Samples were subsequently boiled for 30 min for enzyme inactivation, and products formed were analyzed by HPLC using a homemade Benson BC, calcium column (300 mm \times 7.8 mm i.d.) at 90 °C (TNO, Groningen, The Netherlands) connected to a refractive index detector. A mobile phase of 100 ppm Ca²⁺–EDTA in demineralized water at a flow rate of 0.2 mL/min was used.

RESULTS

Maximizing CGTase Hydrolytic Activity by Saturation Mutagenesis. Both crystal structure data and mutational studies have shown that specific residues in the acceptor sugar binding subsites +1/+2 are essential for the cyclodextrin forming activity of CGTase (7, 22). The A231 and F260 residues of the +1 and +2 acceptor subsites (Figure 1) are strongly conserved in CGTases, but are replaced by various other amino acids in other GH13 enzymes (Table 1). Both residues are known to interact with the substrate during the final stages of the cyclization process (7), giving a strong indication as to where to confine the search in effectively altering the reaction specificities of CGTases (Table 1). In order to increase the hydrolytic potential of Tabium CGTase we constructed two libraries, A231X and A231X/F260X. Screening of 320 clones of the A231X library

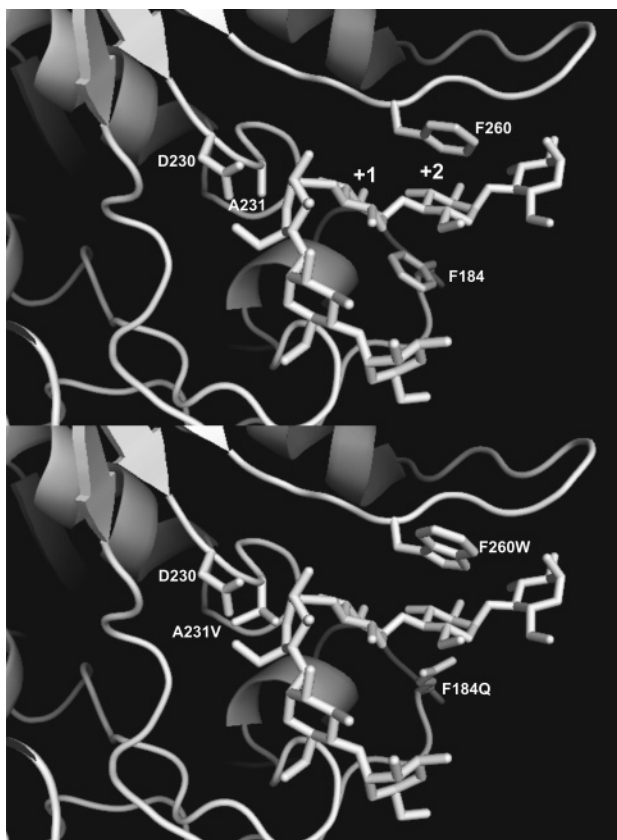


FIGURE 1: Catalytic core region of Tabium CGTase with bound maltohexaose inhibitor. Upper panel displays the wild-type residues A231, F184, and F260 at acceptor subsites +1 and +2, which were targeted for saturation mutagenesis (crystal structure 1A47 from the Protein Data Bank). Lower panel shows a model of one of the triple combinations of mutations selected, namely, A231V/F260W/F184Q. Also included is the catalytic nucleophile D230. Figure 1 was created using PyMOL (46).

led to identification of seven variants with a much higher hydrolytic activity. Of these, three were randomly picked and sequenced, revealing an A231V mutation in each case. This effect of the A231V mutations is in agreement with the equivalent mutation (A230V) in *Bacillus circulans* strain 251 CGTase (22). When screening 720 clones of the A231X/F260X library, four clones with elevated hydrolytic capabilities were selected and sequenced providing an identical double mutation in all instances, namely, A231V/F260W. This double mutant increased the hydrolytic activity of the enzyme from 67 s^{-1} up to 715 s^{-1} (Table 2).

Minimizing Cyclization Activity of CGTase A231V/F260W. One of the main objectives of this study was the evolution of CGTase into a starch hydrolase, making it essential to minimize the relatively high remaining cyclodextrin forming activity of the A231V/F260W mutant. Saturation mutagenesis was therefore carried out at the +2 acceptor subsite residue Phe184, using the double mutant A231V/F260W as parent. Phe184 was chosen because of its pivotal role played in the cyclization process (7). This residue is conserved in CGTases but is known to be replaced mainly by other hydrophobic residues in some well-known α -amylases such as pancreatic (23) and TAKA α -amylase (24) (Table 1). Screening of 300 clones of the A231V/F260W/F184X library yielded several variants combining high hydrolytic activity and low cyclization activity. Sequencing of four positive clones led to the identification of two new mutations, F184H and F184Q, both

in the A231V/F260W background. The A231V/F260W/F184Q mutant displayed a 350-fold reduction in cyclization activity and has the highest hydrolytic activity for a CGTase reported to date, close to that of highly active α -amylases (Table 2). In agreement with the high hydrolysis/cyclization ratios of the triple CGTase mutants compared to wild-type, product analysis after prolonged incubation with starch confirmed that the cyclodextrin forming capacity of the mutants was virtually abolished (Figure 2).

Hydrolysis of G5-pNP. Alpha-amylases generally have a high catalytic efficiency in hydrolysis of the modified saccharide G5-pNP (25). It was therefore of interest to investigate whether the CGTase mutants also had an altered turnover rate or affinity for this substrate, compared to wild-type enzyme. The mutant enzymes indeed displayed increased k_{cat} values for hydrolysis of G5-pNP, with decreased K_M values (Table 3). The selected mutants thus not only possessed a greater hydrolytic activity on starch but also displayed an increase in catalytic efficiency for hydrolysis of this defined substrate, 200-fold in the case of mutant A231V/F184H/F260W.

Generation of Hydrolytic Mutants with epPCR. To construct an epPCR library with a suitable number of mutations, favorable conditions were first determined by amplifying the *Tabium cgt* gene at fifteen different MnCl_2 concentrations. The PCR products were cloned into expression plasmid pCScgt-tt, transformed into *E. coli* MC1061, and plated on LB plates. 192 colonies were picked per MnCl_2 concentration used in the epPCR and inoculated overnight in 96-well microtiter plates. Clones were then analyzed for β -cyclodextrin forming activity. The percentage of active clones decreased from 100% to 4% with MnCl_2 concentrations increasing from 0 to 0.3 mM. We chose MnCl_2 concentrations of 0.005 mM and 0.01 mM as the optimal conditions for the epPCR reactions, with between 80 and 90% of the variants retaining β -cyclization activity. Over 12,000 CGTase clones subsequently were screened for increased starch hydrolytic activity (CGTase is a 683 amino acid protein), using wild-type and mutant A231V/F260W as controls. Selection followed by sequencing of seven selected mutants with strongly enhanced starch hydrolytic activity resulted in identification of the F260L (6x) or A231T (1x) mutations. Randomly chosen clones from mutants displaying a small increase in hydrolytic activity had in all cases a K233R substitution. The overall hydrolytic activity of the K233R mutant remained low despite the 3-fold increase in the hydrolysis/cyclization ratio compared to wild-type. However, both the A231T and F260L mutants showed significantly increased hydrolytic activity with a 16- and 23-fold increase in hydrolysis/cyclization ratio respectively (Table 2).

DISCUSSION

Screening large mutant libraries can often prove both expensive and laborious without automation. A large fraction of the beneficial mutations generally appear in the vicinity of the catalytic site, as revealed by analyzing the results of several laboratory evolved enzymes (26). This indicates that, provided prior structural and mechanistic knowledge is available, the mutant library size may be limited by targeting specific residues of the active site area of enzymes, without severely compromising the identification of positive muta-

Table 1: Alignment of Regions Targeted for Saturation Mutagenesis in Tabium CGTase (F184, A231, and F260 Residues in Bold) with Other Glycoside Hydrolase Family 13 Enzymes

Enzymes	Sequence			Accession no.
	184	231	260	
Cyclodextrin glucanotransferase				
<i>Tabium</i> ^a	GGTD- F SSYED...GIRLDA V KHMP...VFTFGEW L GTN			P26827
<i>Bacillus circulans</i> 251	GGTD- F STTEN...GIRMD V KHMP...VFTFGEW L GVN			P43379
<i>Bacillus ohbensis</i>	GGTD- F SSYED...GIRVDA V KHMS...VFTFGEW L GSG			P27036
<i>Bacillus circulans</i> strain 8	GGSD- F SSLEN...GIRVDA V KHMP...VFTFGEW L GSA			P30920
<i>Klebsiella oxytoca</i>	GG-D- F FQVKN...AIRIDA V KHMD...FFF F GEW F GASA			P08704
<i>Anaerobranca gottschalkii</i>	GGTD- F STYED...GIRVDA V KHMP...VF F FGEW L GKD			Q5ZEQ7
α-amylase				
<i>Bacillus licheniformis</i>	-KAWD E VSNE...GFR L DA V KHIK...MFT V AEY W NDL			P06278
<i>Escherichia coli</i>	QTWHS F NDYIN...GFRV D TA K HVE...FWMT G EAW G HGV			P25718
<i>Aspergillus niger</i>	CLITD W DLTM...GLR I DS V LEVQ...VYCV G EIDNGNP			P56271
<i>Saccharomycopsis fibuligera</i>	CLIS N YDDQ A Q...GLR I DS A KHVD...VYS V GEV F QGD P			P21567
<i>Aspergillus oryzae</i>	CFIQ N YEDQ T Q...GLR I DT V KHVQ...VY C IGEV L DGD P			P10529
Porcine pancreas	GGIES N DPYQ...GFR I DA S KH M W...PF I FQ E V I DL G G			P00690
Neopullulanase				
<i>Bacillus stearothermophilus</i>	G--IT I YLTP...GWRLD V ANEID...VYIL G EI H HDAM			P38940
Amylopullulanase				
<i>Thermoanaerobacter ethanolicus</i>	GTYEG W GFDS...GWRLD V ANEIA...AP M IAEL W GDAS			P38939

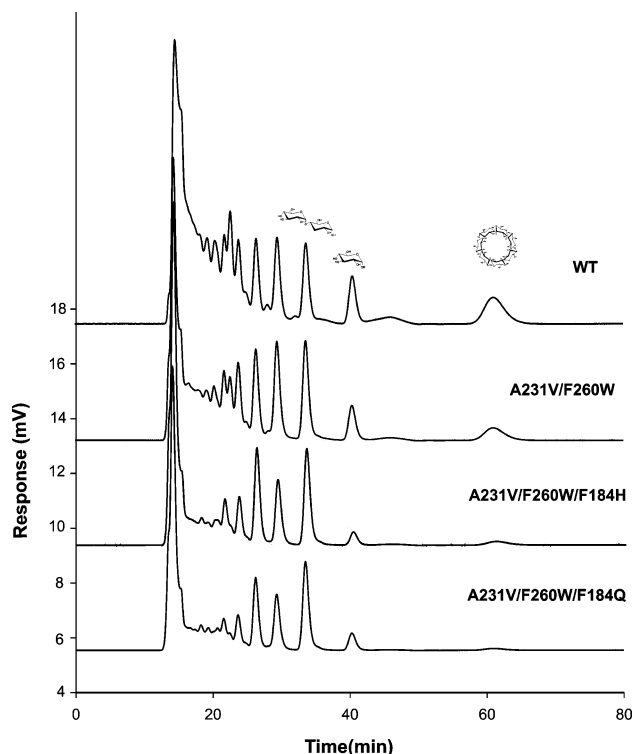
^a *Thermoanaerobacterium thermosulfurigenes*.Table 2: Hydrolysis and Cyclization Activities of Wild-Type Tabium CGTase, and Acceptor Subsite Mutants Derived^a

enzyme	k_{cat} (s ⁻¹)		hydrolysis/ cyclization
	cyclization	hydrolysis	
CGTase			
<i>T. thermosulfurigenes</i> EM1			
wild-type	316 ± 6	67 ± 5	0.2
A231V	30 ± 5	205 ± 12	6.8
A231T	51 ± 2	170 ± 9	3.3
K233R	154 ± 4	86 ± 5	0.6
F260W	150 ± 11	70 ± 5	0.5
F260L	45 ± 2	212 ± 4	4.7
A231V/F260W	54 ± 5	715 ± 9	13.2
A231V/F260W/F184H	2 ± 0.3	485 ± 20	242
A231V/F260W/F184Q	0.9 ± 0.1	730 ± 19	811
α -Amylase			
<i>B. licheniformis</i>	ND ^b	1964 ± 105	
<i>A. niger</i>	ND ^b	1905 ± 105	

^a For comparison, starch hydrolytic activities of two α -amylases are shown. ^b Nondeterminable.

tions (26). However, by applying epPCR, the possibility remains of identification of even more effective mutants, located outside of the catalytic site region. It was therefore of interest to investigate which directed evolution approach would prove most effective in the evolution of CGTase into an α -amylase like enzyme.

Several directed evolution studies have found effective mutations distant from the catalytic core (27, 28), e.g., in the case of enhanced enantioselective *Pseudomonas fluorescens* esterase mutants generated by random mutagenesis (29). However, mutations closer to the catalytic core appear to have a greater effect on the selected catalytic activity (30–33). These residues for mutagenesis may be selected through a random, rational or combined evolutionary approach. In case of a glutaryl acylase from *Pseudomonas* SY-77, the use of epPCR followed by saturation mutagenesis close to the catalytic core of the enzyme increased the substrate selectivity for adipyl-7-aminodesacetoxycephalosporanic acid over 200-fold (34, 35).

FIGURE 2: HPLC elution profiles of CGTase proteins (65 nM) incubated with 10% (w/w) starch for 24 h. The β -cyclodextrin peak is indicated by the circular saccharide. Also the elution positions of glucose and maltose are indicated, by the mono- and disaccharide, respectively.

In our effort to change CGTase into an α -amylase we separately applied both the semirational approach of saturation mutagenesis to the acceptor sugar binding region, and epPCR of the entire Tabium *cgt* gene. Combined saturation mutagenesis at the A231/F260 positions resulted in generation of a unique double mutant (A231V/F260W), not possible by epPCR because the F260W mutation requires two nucleotide substitutions. Furthermore, both mutations were shown to work in a synergistic manner as the single F260W mutant alone had no effect on the hydrolytic activity of the

Table 3: Kinetic Parameters of the *p*-Nitrophenol Liberating Reaction of Wild-Type and Mutant CGTases with G5-pNP

enzyme	k_{cat} (s^{-1})	$K_{\text{M,G5-pNP}}$ (mM)	$k_{\text{cat}}/K_{\text{M,G5-pNP}}$ ($\text{s}^{-1} \text{mM}^{-1}$)
wild-type	3.1 ± 0.2	19.7 ± 3.2	0.16
A231V	23.5 ± 0.4	8 ± 0.4	2.9
A231V/F260W	49.3 ± 1.6	6.4 ± 0.7	7.7
A231V/F260W/F184Q	76.6 ± 1.2	$3.14 \pm .2$	24.4
A231V/F260W/F184H	58.25 ± 1.1	1.8 ± 0.1	32.36

enzyme (Table 2). The semirational approach thus was shown to be successful in the generation of mutant “CGTase” proteins with high hydrolytic activity.

However, the question remained whether random mutagenesis, encompassing the entire *cgt* gene, might even be more effective in generating mutants with higher hydrolytic activity than saturation mutagenesis. If so, were these mutations located in the vicinity of the acceptor subsites, or elsewhere in the CGTase protein? All three positive CGTase mutations identified by epPCR approach were located at the acceptor subsites +1/+2. The two most effective mutations, A231T and F260L, were at residues selected for saturation mutagenesis, highlighting the profound effect of these residues on the hydrolysis/cyclization ratio of the enzyme. However, to be successful in identifying positive mutants from epPCR libraries, a diverse population of sufficient size is required to encompass the majority of possible mutations throughout the protein. Indeed, screening epPCR libraries of limited size, as in the case of the esterase from *Pseudomonas* sp. with less than 300 clones, resulted in identification of distant mutations with low effects on enantioselectivity (26, 29). Screening large libraries generated by random mutagenesis, in contrast, allowed identification of the most effective mutations in the vicinity of the active site, as shown by selection of our hydrolytic CGTase variants. This view is supported, although preliminary, by screening of large libraries in the evolution of an epoxide hydrolase (30) and a glutaryl acylase (35) which identified mutations close to the catalytic core region.

Effect of Mutations at Acceptor Sugar Binding Subsites +1 and +2 of CGTase. The A231/F260/F184 residues at the +1/+2 subsites are completely conserved in CGTases but are replaced by other amino acids at the same position in α -amylases (36) (Table 1). Mutations at these subsites have previously highlighted this acceptor region to be essential in the determination of the reaction specificities of GH clan H enzymes (19, 37–42). The drastic reduction of cyclization activity by these moderately bulkier substitutes A231T/V, A231V/F260W, may be explained by the inefficiency of the final step of the transglycosylation reaction. All CGTase reactions start with substrate binding, followed by cleavage of the glycosidic bond, yielding a covalent intermediate (Figure 3). The covalent intermediate is then transferred to an acceptor molecule, with the type of acceptor determining the reaction specificity of the enzyme. The use of water results in hydrolysis while in the cyclization reaction the covalent intermediate is attacked by its own nonreducing end. The side chains of A231/F260 are located close to the substrate bound at acceptor subsites +1/+2 (Figure 1). The larger valine and threonine side chains introduced at the A231 position are likely to cause steric hindrance with the incoming acceptor sugar, thus obstructing it from its ideal docking

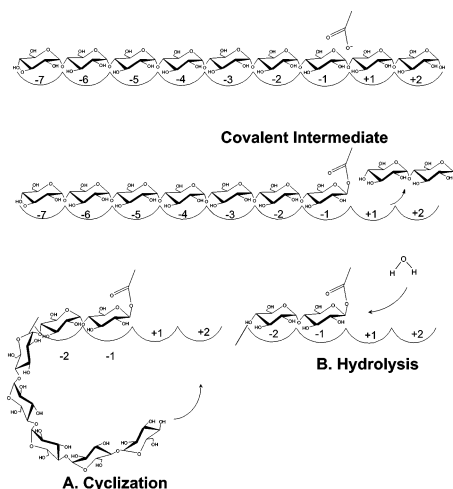


FIGURE 3: Model of reactions catalyzed by CGTase. A linear saccharide chain binds to the enzyme with subsequent bond cleavage between subsite –1 and +1 for the formation of a covalent glycosyl–enzyme intermediate. In the second reaction step the covalently bound oligosaccharide is transferred to an acceptor molecule, *e.g.*, (A) the nonreducing end of a saccharide chain for cyclodextrin formation, or (B) a water molecule in the hydrolysis reaction.

conformation at the acceptor subsite +1. This may result in an inefficient proton abduction from the nonreducing hydroxyl group of the incoming saccharide acceptor by the acid/base residue of the enzyme (Glu 258), preventing efficient glycosidic bond formation of the cyclization process. Another possibility is that the acceptor sugar, though efficiently deprotonated, is not in an ideal position to carry out a nucleophilic attack on the intermediate, thus lowering the efficiency of glycosidic bond formation. Mutants at the F260 and F184 positions lack the phenyl rings of the phenylalanine residues, which have been shown to be essential in guiding the nonreducing end of the bound saccharide into the acceptor subsites by hydrophobic stacking interactions (43). Substitution of these residues, essential in the cyclization process, may further distort or misalign the acceptor sugars, resulting in almost complete loss of transglycosylation activity (Table 2). These CGTase variants, however, still retain a high efficiency of glycosidic bond cleavage, with the glycosyl–enzyme intermediate now collapsing by the nucleophilic attack of the oxygen atom of water. The high hydrolysis rate of the mutants may require an activation of the nucleophilic potential of the water oxygen by amino acids in the active site, to be identified in further investigations.

Saturation and epPCR mutagenesis both proved successful in the generation of hydrolytic mutants of CGTase. But the semirational mutagenesis approach enabled the accumulation of synergistic mutations A231V/F260W/F184Q, resulting in an α -amylase like mutant with virtually no cyclization activity. Only three amino acid mutations, all located at the acceptor substrate binding site, are required to evolve CGTase from a cyclodextrin producing transglycosylase into a highly active starch hydrolase (*i.e.*, an α -amylase). This is a remarkable result since CGTase and α -amylase enzymes share less than 30% amino acid sequence identity. This triple mutant highlights the relative ease by which specificities of glycoside hydrolase family 13 enzymes can be interchanged, as most amino acid differences between these enzymes seem to have no effect on specificity. This view is supported by

the conversion of a maltogenic amylase (Novamyl) into a CGTase through a five residue loop deletion followed by a double mutation (44). Also a single amino acid exchange at the active site of Tabium CGTase and acarviosyl transferase (ATase) was sufficient to interchange reaction specificities of the two enzymes (45). Taken together, these results suggest that various glycoside hydrolyase family 13 members have diversified by introduction of a limited number of mutations. The present study demonstrates that it may be easier to interchange reaction specificities than previously thought.

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