Evolution toward small molecule inhibitor resistance affects native enzyme function and stability: generating acarbose insensitive cyclodextrin glucanotransferase variants

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Running head: Evolutionary constraints of inhibitor resistant variants.
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Small molecule inhibitors play an essential role in the selective inhibition of enzymes associated with human infection and metabolic disorders. Targeted enzymes may evolve toward inhibitor resistance through selective incorporation of mutations. Acquisition of insensitivity may, however, result in profound devolution of native enzyme function and stability. We therefore investigated the consequential effects on native function and stability by evolving a cyclodextrin glucanotransferase (CGTase) enzyme toward insensitivity to the protein’s small molecule inhibitor, acarbose. Error-prone PCR mutagenesis was applied to search the sequence space of CGTase for acarbose insensitive variants. Our results show that all selected mutations were localized around the active site of the enzyme, and in particular at the acceptor substrate binding sites, highlighting the regions importance in acarbose inhibition. Single mutations conferring increased resistance, K232E, F283L and A230V, raised IC50 values for acarbose between 3,500- to 6,700-fold compared to wild-type CGTase, but at a significant cost to catalytic efficiency. In addition the thermostability of these variants was significantly lowered. These results reveal not only the relative ease by which resistance may be acquired to small molecule inhibitors, but also the considerable cost incurred to native enzyme function and stability, highlighting the subsequent constraints in the further evolutionary potential of inhibitor resistant variants.

Many small molecule inhibitors play a central role in today’s treatment of human diseases targeting an essential structure or process of the bacterium, virus or host’s cell itself. Bactericidal antibiotics including aminoglycosides, macrolides and tetracyclines are all potent inhibitors of bacterial protein synthesis (1-4). Penicillins along with glycopeptides target peptidoglycan synthesis of the bacterial cell wall (5;6). Numerous small molecule nucleoside and nucleotide inhibitor analogs have been synthesised to target the essential enzymes of the human immunodeficiency virus (HIV) (7). The powerful mitotic anti-tumour inhibitor, taxol, promotes the assembly and hyper-stabilization of microtubules in the treatment of breast, lung and ovarian cancers (8;9). However, resistance to all of these effective inhibitors, due to intrinsic or acquired immunity, seems to be a mere formality (10;11). The target cell may apply numerous counteractive measures to prevent the fatal actions of inhibitors. Multidrug efflux pumps are extremely effective in the removal of inhibitors from the cell in gram negative bacteria (12). Inhibitor modification by enzymes such as aminoglycoside acetyltransferases and phosphotransferases render aminoglycoside antibiotics ineffective (2). Another form of resistance, involves incorporation of single or multiple mutations at the inhibitors target site, ultimately leading to non-adherence and lack of inhibitor potency. Mutated variants must however, preserve cell function while acquiring resistance. Studies of clinical isolates have shown that the biological cost of native enzyme function by the acquisition of antibiotic resistance is a main determinant of both the rate and extent of resistance development under a given antibiotic pressure (11). While compensatory mutations may aid in lowering this
biological cost there is ultimately a price to be paid in the initial acquisition of this newly attained function (13-15). To investigate the delicate balance between this newly attained function and native function and stability, we have evolved a cyclodextrin glucanotransferase (CGTase) enzyme toward resistance to the protein’s small molecule inhibitor, acarbose (Fig. 1). CGTase is a well-studied model enzyme for the glycoside hydrolase family 13 (GH13) (16-18) catalysing the formation of \( \alpha-(1,4) \)-linked oligosaccharides (cyclodextrins) from starch (Fig. 1) and is strongly inhibited by the small molecule acarbose (19;20). To evolve this enzyme toward acarbose insensitivity we applied directed evolution, introducing random mutations throughout the \( cgt \) gene by error-prone (ep) PCR. CGTase variants were subsequently screened for native cyclodextrin forming activity in the presence of high acarbose concentrations. Our results demonstrate the relative ease at attaining acarbose resistant CGTase mutants, increasing the IC\(_{50}\) value for acarbose up to 6,700-fold. However, detailed analysis of the insensitive variants highlights the conflicting compromise between native and newly attained enzyme function and subsequent impact on protein stability.

**Experimental Procedures**

*Bacterial strains, plasmids and growth conditions– Escherichia coli* strain MC1061 was used for DNA manipulations and library screening. Plasmid carrying strains were grown on LB medium at 37\(^\circ\) C in the presence of kanamycin (50 \( \mu \)g/mL for *E. coli* and 5 \( \mu \)g/mL for *Bacillus subtilis*). *Bacillus circulans* 251 (BC251) CGTase proteins were produced from plasmid pDP66k- using *B. subtilis* strain DB104A as host, as described (19). Purity and molecular weight were checked by SDS-PAGE. Enzyme concentrations were determined using the Bradford reagent from Bio-Rad (München, Germany) and bovine serum albumin as standard.

**Saturation and site-directed mutagenesis –** Mutants were constructed in pDP66k- as described (21) and verified by DNA sequencing (BaseClear, Leiden, the Netherlands). Construction of the single mutants A230V, K232E, F283L, I61V, D313E and D319E was carried out using the following oligonucleotides: 5’-GC CTG CTT GAT CGT TTT GC-3’ (F283L); 5’-TGG ATG CGG TG AAG CAT A-3’ (K232E); 5’-AC AAA GTC AAC GAC GG TTA C-3’ (I61V); 5’-TCC GCA GCC GAA TAC GCC CA-3’ (D313E); 5’-CGC ATG GAT GTG GTG AAG CAT ATG CCG TTC G-3’ (A230V); 5’-AT GAA CAG GTG ACG TTC ATC-3’ (D319E). Underlined regions of oligonucleotides indicate where the nucleotide substitution was introduced. Double mutants H140Q/F283L, A230V/F283L, and A230V/H140Q were subsequently constructed using H140Q and A230V oligonucleotides with F283L and H140Q mutants as PCR template. H140 was replaced by all nineteen other amino acid residues by site-saturation mutagenesis, using the oligonucleotide: H140X, 5'-TTT GCC CCG AAC NNS ACG TC-3’. The underlined region indicates where the nucleotide substitutions were introduced. N is A+G+C+T, S is G+C and X is any amino acid residue.

**Error-prone PCR mutagenesis–** The \( cgt \) gene was amplified from pDP66k- with the primers F1 (XhoI), 5’-GCG CCG GAT ACC TCG AG 3’ and Rev1 (KpnI), 5’-CCA ATT CAC GTT AAT GGT ACC GGC GCC GCT GGA CCG-3’. The XhoI and KpnI restriction sites introduced (underlined) into pDP66k- resulted in V6S (N-terminus) and A678G (C-terminus) mutations, which had no effect on the catalytic properties of the enzyme (22). PCR mixtures (50 \( \mu \)l) contained: 1x Taq DNA polymerase buffer, 1 mM MgSO\(_4\), 0.25 mM MnCl\(_2\), 0.6 mM of each dNTP, 0.07 \( \mu \)M of each primer, 20 ng pDP66k- and 2.5 units Taq DNA polymerase (Roche). PCR reactions were performed for 25 cycles: 30 sec 94\(^\circ\) C, 40 sec 54\(^\circ\) C, and 2 min 72\(^\circ\) C. The PCR products were restricted with XhoI and KpnI, and the resulting fragment (2100 bp) was extracted from agarose gel (QIAquick Gel Extraction Kit; Qiagen) and cloned in pDP66k-, replacing the wild-type \( cgt \) gene.

**Gene shuffling –** DNA shuffling of the single variants (A230V, F283L, K232E, H140Q) and wild-type BC251 \( cgt \) genes was carried out using an adapted version applied by Kikuchi et al. (23;24). Wild-type and mutant genes were amplified using the flanking primers FLKF, 5’-GGA CAA GCC TGG AAT TCA-3’ and FLKR,
5'-CCG AAG CTT GCT CAA TCA-3'. PCR products were subsequently diluted to a concentration of 84 µg/mL before being pooled. Separate overnight restriction digestions of the pooled variants by MwoI, MspI, TaqI/EcoRII, NciI/Sau3AI and HaeIII/NciI, were followed by thermal enzyme inactivation. DNA fragments were reassembled in the following PCR cycles lacking primers: 96° C, 90 s; 35 cycles of (94° C, 30 s; 65° C, 90 s; 62° C, 90 s; 59° C, 90 s; 56° C, 90 s; 53° C, 90 s; 50° C, 90 s; 47° C, 90 s; 44° C, 90 s; 41° C, 90 s; 72° C, 4 min); 72° C, 7 min; 4° C 10 min. One µl of this reaction was used for generation of the full length cgt gene by PCR using the F1 and Rev1 primers (mentioned above). The resulting full length gene products were cloned into the expression vector to obtain the second generation library.

Selection of acarbose insensitive CGTase variants- E. coli MC1061 cells were transformed with the epPCR library and plated on LB agar plates. Resulting colonies were transferred to 200 µL of LB medium in 96-well microtiter plates using the Q-pix (Genetix, New Milton Hamshire, U.K.) and incubation overnight (750 rpm) at 37° C. From each well, 25 µL culture was transferred to a second 96 well plate containing 25 µl per well of bacterial protein extraction reagent (Pierce, Rockford, IL) to lyse the cells. Subsequently, 200 µL of 1% (w/v) Paselli SA2 starch with 250 µM acarbose (Serva Electrophoresis, Heidelberg, Germany) in 10 mM sodium citrate buffer (pH 6.0) was added, and the microtiter plates incubated at 50° C for 5 h in an oven. Under these conditions wild-type CGTase displayed no detectable cyclization activity. The amount of β-cyclodextrin formed was measured by the addition of 10 µL of the reaction to 100 µL phenolphthalein solution before reading absorbance at 552 nm (25).

Enzyme assays– All CGTase enzyme assays (initial rates) 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 hydrolysed potato starch with an average degree of polymerisation of 50 (Paselli SA2; AVEBE, Foxhol, The Netherlands). The amount of β-cyclodextrin formed was measured by the addition of 10 µL of the reaction to 100 µL phenolphthalein solution before reading absorbance at 552 nm (25).

Differential Scanning Calorimetry (DSC)– Thermal unfolding of (mutant) CGTases was measured using a MicroCal VP-DSC microcalorimeter (MicroCal Inc., Northhampton, Massachusetts, USA) with a cell volume of 0.52 mL. Experiments were performed at a scan rate of 1° C/min at a constant pressure of 2.75 bar. Samples were degassed prior to scan. The enzyme data was fitted to a four parameter non-linear regression function using SigmaPlot 10 software (Systat): E = max + ((min-max)/(1+C/IC50)k)) + max, where min = minimum response, max = maximum response, C = acarbose concentration, IC50 = acarbose concentration causing half maximum β-cyclization activity and k = curve slope.

Disproportionation activity was measured as described (19), using 0.06 - 0.6 nM enzyme, 0.05 - 3 mM 4-nitrophenyl-α-D-maltoheptaoside-4-6-O-ethylidene (pNPG7; Megazyme, Wicklow, Ireland) as donor substrate and 0.05 - 3 mM maltose as acceptor substrate. The 4-hydroxyl of the donor substrate pNPG7 is blocked thereby preventing that the donor substrate being used as acceptor substrate.

Fitness of epPCR library – Competent E. coli MC1061 cells were transformed with the epPCR plasmid library and the entire transformation (approximately 2 million transformants) was used to inoculate 2 L LB media containing 50 µg/mL kanamycin. Following overnight growth at 37° C, cells were collected by centifugation. Cells were broken by French Press (10,000 p.s.i), and cell debris removed by ultracentrifugation at 4° C for 30 min at 15,000 g. CGTase proteins were subsequently purified as previously described (22).

HPLC analysis- Formation of cycloextrins from starch (10% (w/v) Paselli SA2 in 10 mM sodium citrate buffer pH 6.0) was analysed by incubating the starch for 54 h with 13 nM of wild-type and 65 nM of mutant proteins (A230V, H140Q, K232E, F283L and A230V/H140Q). Samples were taken at regular time intervals and subsequently boiled for 30 min for enzyme inactivation. Products formed were analysed on a homemade Benson BC, calcium column (300 mm x 7.8 mm ID) at 90° C 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.
concentration used was 6.9 µM in 10 mM sodium acetate buffer, pH 5.5 (26).

RESULTS

Generation of acarbose insensitive mutant CGTase proteins- Random genetic diversity was created by error-prone PCR amplification of the cgt gene in the presence of 0.25 mM MnCl₂. Under these conditions 90% of the E. coli transformants retained starch degrading activity as detected by the formation of halos surrounding the colonies on starch/agar plates. The fitness of the epPCR library was 60% regarding the initial β-cyclization activity and the IC₅₀ value for acarbose was 1.2 µM, nearly identical to that of the wild-type enzyme (Table 1). Twelve thousand variants of the library were screened for decreased inhibition by acarbose. Fifty-six variants retained β-cyclization activity in the presence of 250 µM of the acarbose inhibitor. Sequencing of 13 variants revealed the presence of A230V (9x), K232E (2x) and F283L (2x) mutations. Also I61V, D313E and D319E mutations were found, but only in combination with those mentioned. Construction and characterisation of the purified proteins of the single mutations I61V, D313E and D319E mutations were found, but only in combination with those mentioned. Construction and characterisation of the purified proteins of the single mutations I61V, D313E, D319E, A230V, K232E and F283L, demonstrated that the A230V, K232E and F283L mutations were responsible for CGTase insensitivity to acarbose inhibition (Table 1). Surprisingly, no H140 mutant was identified from the screening as this histidine residue has been shown to be important for the strong inhibition by acarbose in Bacillus sp. 1011 and Thermoanaerobacterium thermosulfurigenes EM1 CGTases (27;28). H140 was therefore targeted by saturation mutagenesis. Screening of the 288 clones from the H140X library, yielded six variants that formed β-cyclodextrins in the presence of 250 µM of the acarbose inhibitor. Sequence analysis of 13 variants revealed the presence of a His to Gln substitution in each case. Shuffling and construction of double mutant CGTase variants– In an effort to further decrease the inhibitory effects by acarbose, while retaining or increasing native β-cyclization activities, DNA shuffling of the selected variants from the error-prone library was carried out. Over 8,000 clones were screened at an increased concentration of 500 µM acarbose, however, no improved variants were found. To investigate whether combinations of mutations selected in the first round had additive effects for CGTase insensitivity toward acarbose, the double mutants A230V/F283L, A230V/H140Q and F283L/H140Q were constructed. Only the A230V/H140Q mutant remained functional in β-cyclization production, with lower acarbose resistance levels compared to the single mutants generated by epPCR (Table 1). This may explain the lack of identification of better performing mutants from shuffling.

IC₅₀ values of wild-type and mutant CGTases- Measurement of β-cyclization activity in the presence of varying concentrations of acarbose revealed that mutations at the acceptor subsites of CGTase had a profound effect on inhibitor insensitivity, raising IC₅₀ values by 3,500- to 6,700-fold compared to wild-type (Table 1). Mutation H140Q, located at the -1 donor subsite, had the least effect on inhibitor resistance though still increasing the IC₅₀ value over 1,600-fold compared to wild-type (Table 1).

Catalytic properties of wild-type and mutant CGTases– The β-cyclization rates of the selected mutations were compromised (Table 1). The F283L, K232E and A230V/H140Q variants showed comparable decreases of approximately 10-fold in the initial β-cyclization rates compared to wild-type, while the A230V mutation was most severely affected with an almost 40-fold reduction in activity. Product analysis also revealed a 3.4-fold reduction in cyclodextrin production for the A230V mutant, with a large increase in short linear saccharide formation compared to wild-type (not shown). All other mutants formed approximately the same amount of β- and γ-cyclodextrins as wild-type, with lowered production of α-cyclodextrin (Fig. 2). How the mutations at the subsites -1/+1/+2 alter cyclodextrin product specificity is not yet clearly understood (29).

Measurements of the catalytic efficiency (kcat/KM) for processing of the maltoheptaoside substrate pNPG7 in the disproportionation reaction with maltose as acceptor substrate, revealed a 5-fold (F283L) to 18-fold (H140Q and K232E) reduction (Table 2). Surprisingly, the catalytic efficiency of the A230V/H140Q combination is much higher than that of its single mutant counterparts. The KM values for the acceptor substrate maltose were not significantly altered by most mutations, with only the H140Q mutant not significantly altered by most mutations, with only the H140Q mutant
displaying a 2-fold decrease compared to wild-type (Table 2).

Stability of wild-type and mutant CGTases- To investigate whether the mutations selected for their capacity to minimize acarbose inhibition affected the stability of the enzyme, the CGTase variants were denatured by heat using differential scanning calorimetry (DSC). Both the wild-type and mutant proteins displayed irreversible thermal unfolding patterns. All mutants were significantly affected in stability, lowering the apparent melting temperature between 7 and 11 °C (Fig. 3).

DISCUSSION

Mechanism and effects of acquired inhibitor resistance- As a general trend, most resistance conferring mutations are located throughout the active site due to the substrate mimicking nature of small molecule inhibitor (Table 3). As active sites have primarily evolved for reaction specificity and rate enhancement, introduction of such mutations is expected to have a negative effect on the enzyme’s catalytic efficiency. A trade-off between enzyme function and inhibitor resistance is indeed observed for enzymes with resistance conferring mutations (14;15;30-35) (Table 3). Directed evolution of our model enzyme, BC251 CGTase, also resulted in most effective resistance conferring mutations (A230V, K232E and F283L) clustered in and around the active site (Fig. 4). All three residues (A230, K232 and F283) are conserved amongst CGTases (Supplementary Table S1), indicating their important role in enzyme catalysis and reaction specificity. Replacement of such essential residues yielded variants with severely compromised catalytic function (Tables 1 & 2).

Comparison of the crystal structures of CGTase mutants A230V (BC251) and F283L (Bacillus sp. strain 1011) with their respective wild-types, revealed a number of structural differences responsible for increased insensitivity to acarbose. The larger valine side-chain of the A230V mutant partially blocks the +1 acceptor subsite, obstructing the inhibitor from attaining an ideal binding conformation at the active site, resulting in weaker inhibitor binding. Such side chain intrusion would also affect substrate binding, explaining the strongly reduced catalytic rates of this mutant (Table 1 & 2). In wild-type BC251 CGTase, K232 forms hydrogen bonds via its Nζ atom to the O2 and O3 atoms of linear oligosaccharides and acarbose at the +2 subsite (Fig. 4) (36;37). These interactions are not possible in the K232E mutant, leading to weaker binding of acarbose and thus reduced inhibition. While the F283 has no direct interactions with bound substrate/inhibitor, F283 is important for stabilisation of the +2/+3 acceptor subsite region. In a F283L crystal structure of Bacillus sp. strain 1011 CGTase, the isotropic temperature factors of Cα atoms of acceptor subsite +2/+3 residues (residues 259-269) increased from 20 to 30 Å² compared to the wild-type structure. The structure of this region, which is highly similar to that of BC251 CGTase, is therefore more flexible in the F283L mutant than in the wild-type (38). Greater flexibility of the acceptor subsites would contribute to a less favourable, weaker binding of substrate/inhibitor at the active site, resulting in reduced catalytic rates and increased inhibitor insensitivity (Table 1 & 2).

Targeted by saturation mutagenesis, the highly conserved H140 GH13 residue forms a hydrogen bond via its side-chain to the valienamine moiety of acarbose at donor subsite -1 (39;40). Substitution of this residue by glutamine may alter or disrupt this specific enzyme-inhibitor interaction leading to reduced inhibition by acarbose. Interestingly, this residue is replaced in some GH13 acarbose resistant glucanotransferases, suggesting an evolutionary role for acquired resistance (Supplementary Table S1) (28;41).

Our most effective mutations reduce enzyme inhibitor sensitivity by direct obstruction (A230V), removal of essential bonding interactions (K232E, H140Q), or by influencing secondary binding effects between enzyme and inhibitor (F283L). Furthermore, mutations that confer for higher magnitudes of inhibitor insensitivity generally have a greater detrimental effect on native functionality (Table 3). The A230V mutation, selected HIV-protease and xylanase variants, display large increases in inhibitor insensitivity, but not without substantial cost to native catalytic efficiency (Table 3). Thymidylate synthase, isoleucyl-tRNA synthetase and HIV protease variants however, retain greater levels of functional activity but also display lower fold increases in inhibitor resistance over their
respective wild-types (Table 3). Few inhibitor insensitive variants persist with high levels of inhibitor resistance and native catalytic rates. This observation may explain the lack of identification of improved variants from the second round of directed evolution of resistant variants by gene shuffling. While new improved acarbose insensitive mutants may have been generated at increased inhibitor concentrations, their effect on enzyme function was simply too damaging for detection and selection by the screening method applied.

Protein stability of evolved variants – In addition to reaction specificity and catalytic rates, active site mutations may also have a profound effect on enzyme stability (42;43). As mentioned active sites are flexible, primed for substrate recognition and rate enhancement and so sacrifice the stability ethos of the remaining protein scaffold (44;45). Mutations such as F283L, introducing greater flexibility within this region, may destabilise the enzyme by increasing active site strain or loss of favourable interactions (42) (Fig. 3). Replacement of F283 with leucine is thought to remove stabilising CH-π interactions with residue F323 and acceptor subsite +2 residue, F259 (38). The A230V mutation may affect the structural stability of the enzyme by compromising the structural integrity of a hydrophobic cavity located at the +1 subsite. Removal of essential hydrogen bond stabilising interactions with neighbouring amino acids such as H233 are thought to decrease the stability of the K232E variant (46). The H140Q substitution disrupts the hydrogen bond network between H140 and neighbouring Y100, N139 and T141 residues at the -1 donor subsite (36), resulting in reduced thermostability. Interestingly, the limited number of enzymes evolved towards inhibitor insensitivity, for which stability data is available, also display decreased stabilities (Table 3).

Despite the overall reduction in protein stabilities (Fig. 3), these variants remained functional for extended periods of time (54 h, during the cyclodextrin production assay (Fig. 2)). Mutant libraries were screened at a temperature 14°C below the denaturing temperature of the wild-type enzyme, thus allowing for the identification of acarbose insensitive variants. Screening of mutant libraries close to wild-type denaturating temperatures would have compromised the identification of less stable acarbose insensitive variants. Therefore, screening and selection of newly evolved variants from evolution experiments is best performed at lower temperatures, allowing for identification of less stable variants with new or increased specificities. If required, subsequent rounds of directed evolution may be applied to increase the stability of evolved but unstable variants (47). Selection of more stable enzymes for evolution over their less stable counterparts may also circumvent this evolutionary constraint. Recently, Bloom et. al. found it easier to introduce novel enzyme activities into a more thermostable P450 monooxygenase variant than a less stable parent, by applying directed evolution (43).

Compensatory mutations restore native protein function – Emergence of drug insensitive variants usually involves the initial acquisition of mutations impairing native enzyme function and stability (Table 3). To deal with this negative impact proteins may evolve in either of two directions; either back to the ancestral state and become dysfunctional and extinct, or accumulate mutations which compensate for these negative effects while retaining or increasing resistance ability. Such compensatory mutations may have deleterious effects on protein function as a single mutation but neutral when combined with other mutations (48-50). Indeed, the combination of the single A230V and H140Q mutations, constructed by site directed mutagenesis, displayed compensating behaviour toward CGTase function. Initial cyclization rates and overall cyclodextrin production were increased considerably for the double mutant compared to the A230V variant (Table 1 & Fig. 2).

To conclude, we have highlighted the relative ease of attaining highly effective inhibitor insensitive CGTase variants by directed evolution. All selected mutations (H140Q, A230V, K232E and F283L) were found clustered around the active site area. Comparison of our selected CGTase variants with other enzymes evolved towards inhibitor insensitivity highlights the cost and conflict between native enzyme function and level of inhibitor resistance attained. The reduced thermostabilities of acarbose inhibitor resistant CGTase variants may significantly lower the evolutionary potential of the resistant variants, as
it is unlikely that they can accept more destabilizing mutations.

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FOOTNOTES

HL acknowledges financial support from the Netherlands Organization for Scientific Research (NWO). Abbreviations used: BC251, Bacillus circulans strain 251; CGTase, cyclodextrin glucanotransferase; epPCR, error-prone polymerase chain reaction.

FIGURE LEGENDS

**Fig 1.** Binding pattern of acarbose and oligosaccharides in the active site of CGTase. (A) Acarbose consists of a valienamine, linked to 6-deoxyglucose via a nitrogen bridge followed by maltose. (B) Binding pattern of acarbose in the active site of CGTase, with the non-cleavable nitrogen bridge positioned between the -1 and +1 subsites. The valienamine moiety resembles the planar sugar structure of the transition state for increased binding affinity (51). (C) Binding of oligosaccharide substrate in the active site prior to bond cleavage between the -1 and +1 subsites. (D) Final stages of cyclodextrin formation, with the transition state structure depicted at subsite -1. Note the similarity between the transition state structure and the valienamine structure of acarbose. Asp299 is the catalytic nucleophile of the enzyme.

**Fig 2.** Cyclodextrin production (g/L) from 10% (w/v) Paselli SA2 starch by wild-type and mutant CGTases at pH 6.0 and 50°C. α, β and γ cyclodextrins are indicated by white circles, black circles, and squares, respectively.

**Fig 3.** Thermal denaturation curves of wild-type and mutant CGTases as measured with differential scanning calorimetry. The panel gives the apparent melting temperatures of variants.

**Fig 4.** (A) Surface representation of CGTase with highlighted location of resistance conferring mutations (blue). (B) Catalytic core region of B. circulans 251 CGTase with bound maltotetraose. The panel displays the residues H140, A230, K232 and F283, at subsites -1, +1 and +2, found mutated in acarbose insensitive variants (crystal structure 1CXH from the protein data bank). Hydrogen bond interactions between maltotetraose and residues are indicated by dashed white lines. Figure 4 was created using PyMOL (52).
Table 1. Cyclization rates from starch and IC$_{50}$ values for acarbose by wild-type and acarbose insensitive CGTase mutants from Bacillus circulans 251

| Enzyme               | β-cyclization $k_{\text{cat}}$ (s$^{-1}$) | IC$_{50}$ (µM) | Fold-increase$^a$ |
|----------------------|------------------------------------------|----------------|-----------------|
| Wild-type            | 329 ± 3                                  | 1.1            | 1               |
| EpPCR library$^b$    | 195 ± 5                                  | 1.2            | 1.1             |
| A230V                | 9 ± 0.1                                  | 7,370          | 6,700           |
| H140Q                | 79 ± 3                                   | 1,807          | 1,642           |
| F283L                | 35 ± 0.5                                 | 4,197          | 3,815           |
| K232E                | 26 ± 1                                   | 3,930          | 3,572           |
| A230V/H140Q          | 48 ± 2                                   | 1,845          | 1,677           |

$^a$ Fold increase indicates the factor of improvement of variants compared to wild-type based on IC$_{50}$ values.

$^b$ Mixture of mutant CGTases from the epPCR library.

Table 2. Kinetic parameters of the disproportionation reaction of wild-type and mutant B. circulans 251 CGTases with the blocked pNPG7 and maltose substrates.

| Enzyme               | $k_{\text{cat}}$ (s$^{-1}$) | $K_M$, pNPG7 (mM) | $K_M$, maltose (mM) | $k_{\text{cat}}/K_M$, pNPG7 (s$^{-1}$/mM) |
|----------------------|----------------------------|-------------------|---------------------|------------------------------------------|
| Wild-type            | 490 ± 10                   | 0.09 ± 0.01       | 1.2 ± 0.17          | 5,444                                    |
| A230V$^a$            | 45 ± 2                     | 0.30 ± 0.04       | -                   | 150                                      |
| H140Q                | 77 ± 2                     | 0.26 ± 0.01       | 0.48 ± 0.03         | 296                                      |
| F283L                | 136 ± 5                    | 0.11 ± 0.01       | 1.4 ± 0.11          | 1,236                                     |
| K232E                | 84 ± 1                     | 0.27 ± 0.01       | 1.2 ± 0.17          | 311                                      |
| A230V/H140Q          | 144 ± 5                    | 0.09 ± 0.01       | 1.9 ± 0.07          | 1,600                                     |

$^a$ Due to elevated hydrolytic rates of A230V, only hydrolysis of pNPG7 could be measured. The presence of maltose had no effect on the rate of pNPG7 degradation.
Table 3. Effects of evolved variants toward small molecule inhibitor insensitivity, on native enzyme function and stability.

| Enzyme (Inhibitor) | Mutations (Location) | Fold reduction<sup>a</sup> | Cost to native<sup>b</sup> | Ref |
|---------------------|---------------------|---------------------------|--------------------------|-----|
| Thymidylate synthase (5-fluorodeoxyuridine) | T51S ● | 11 | 0.56 | - | (53) |
| HIV reverse transcriptase (DNA aptamer, RT1t49) | N255D/N265D ● | 150 | 0.43 | - | (31) |
| Glutamate-1-semialdehyde aminotransferase (4-amino-5-flouro pentanoic acid) | S163T ● | >16.6 | 0.08 | - | (54) |
| Hepatitis C Virus Serine Protease (Protease Inhibitor BILN 2061) | D168A ● | >200 | 0.167 | - | (55) |
| Taq DNA polymerase (Heparin) | K225E/E388V<sup>c</sup> ● | 260 | 0.72 | 0.2<sup>d</sup> | (56) |
| HIV-1 protease (p2-NC analog) | G48V ● | 27 | 0.55 | 0.35<sup>e</sup> | (57) |
| Isoleucyl-tRNA synthetase (Mupirocin) | H594Y ● | >43<sup>f</sup> | 0.42 | - | (58) |
| HIV-1 protease (Indinavir) | I50V ● | 50 | 0.18 | 0.6<sup>g</sup> | (59) |
| ROB-1 β-lactamase (Clavulanate) | S130G ● | 166 | 0.15 | - | (60) |
| HIV-1 protease (Protease inhibitor BILA2185BS) | L23I/V32I<sup>g</sup> ● | 1,500<sup>h</sup> | 0.003 | - | (61) |
| Xylanase (Aza-sugar inhibitor) | K47A ● | 6,970 | 0.002 | - | (51) |
| Cyclodextrin glucanotransferase (Acarbose) | A230V ● | 6,700 | 0.03 | 7.5<sup>i</sup> | This study |

● Active site. <sup>a</sup>Fold reduction indicates the factor of reduced inhibitor sensitivity of variants compared to wild-type based on IC<sub>50</sub> or K<sub>i</sub> values. <sup>b</sup>Comparison between variants and wild-type based on k<sub>cat</sub>/K<sub>M</sub> for enzymatic activities and indicated thermostability tests. <sup>c</sup>Contains additional mutations D578G/N583S/M747R/K540R. <sup>d</sup>Thermostability – activity half-life (t<sub>1/2</sub>) at 95°C. <sup>e</sup>Ratio of the urea concentrations at half maximal activity. <sup>f</sup>Fold increase in MIC (µg ml<sup>-1</sup>) of mupirocin for Salmonella typhimurium growth. <sup>g</sup>Contains additional mutations M461/I47V/I54M/A71V/I84V. <sup>h</sup>Fold increase of drug concentration giving 50% inhibition of HIV-1 strain IIIB. <sup>i</sup>Reduction in apparent melting temperature (°C) as measured by Differential Scanning Calorimetry.
Figure 1

A. Valinamidine

B. Asp229

C. Asp229

D. Asp229
Figure 2
Figure 3

| Enzyme           | T_m (°C) |
|------------------|----------|
| WT               | 63.8     |
| A230V            | 56.3     |
| H140Q            | 55.6     |
| A230V/H140Q      | 55.2     |
| F283L            | 55.1     |
| K232E            | 52.6     |

T (°C)
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