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The Evolution of Catalytic Efficiency and Substrate Promiscuity in Human Theta Class 1-1 Glutathione Transferase

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Abstract

Theta class glutathione transferases (GST) from various species exhibit markedly different catalytic activities in conjugating the tripeptide glutathione (GSH) to a variety of electrophilic substrates. For example, the human theta 1-1 enzyme (hGSTT1-1) is 440-fold less efficient than the rat theta 2-2 enzyme (rGSTT2-2) with the fluorogenic substrate 7-amino-4-chloromethyl coumarin (CMAC). Large libraries of hGSTT1-1 constructed by error prone PCR, DNA shuffling, or saturation mutagenesis were screened for improved catalytic activity towards CMAC in a quantitative fashion using flow cytometry. An iterative directed evolution approach employing random mutagenesis in conjunction with homologous recombination gave rise to enzymes exhibiting up to a 20,000-fold increase in k_{cat}/K_M compared to hGSTT1-1. All highly active clones encoded one or more mutations at residues 32, 176, or 234. Combinatorial saturation mutagenesis was used to evaluate the full complement of natural amino acids at these positions, and resulted in the isolation of enzymes with catalytic rates comparable to those exhibited by the fastest mutants obtained via directed evolution. The substrate selectivities of enzymes resulting from random mutagenesis, DNA shuffling, and combinatorial saturation mutagenesis were evaluated using a series of distinct electrophiles. The results revealed that promiscuous substrate activities arose in a stochastic manner, as they did not correlate with catalytic efficiency towards the CMAC selection substrate. In contrast, chimeric enzymes previously constructed by homology-independent recombination of hGSTT1-1 and rGSTT2-2 exhibited very different substrate promiscuity profiles, and showed a more defined relationship between evolved and promiscuous activities.

Keywords

enzyme engineering; glutathione transferase; substrate promiscuity; high-throughput screen; directed evolution

INTRODUCTION

Glutathione Transferases (GSTs)§ are critical components of cellular detoxification systems. An important mode of action employed by these protective enzymes is the conjugation of reactive electrophilic compounds to the endogenous tripeptide glutathione (GSH).¹ While

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GSTs exhibit widely diverse functions and amino acid compositions, all soluble classes possess a highly conserved 3-dimensional structure consisting of an *N*-terminal domain with a thioredoxin-type fold and a *C*-terminal α -helical domain.² The *N*-terminal domain is largely responsible for binding and activating the GSH substrate, and the corresponding portion of the active site is typically referred to as the G-site. Likewise, the electrophilic substrate binding pocket is composed of residues found in the *C*-terminal domain or H-site.

In part, due to their conserved modular structure and highly divergent functions/specificities, soluble GSTs have served as useful models for understanding the effect of mutations on enzyme function.^{3,4,5,6,7,8} Recently, fluorescent single cell assays for GST activity have been developed, opening the way for screening of large mutant libraries by flow cytometry.^{9,10} Griswold *et al.* reported the construction of large libraries by homology-independent recombination of the human theta class 1-1 (hGSTT1-1) and rat theta class 2-2 (rGSTT2-2) GSTs.¹¹ The two parental enzymes exhibit drastically different substrate specificities, with rGSTT2-2 having >400-fold higher catalytic efficiency for the conjugation of 7-amino-4-chloromethyl coumarin (CMAC) compared to hGSTT1-1. Flow cytometric screening of the libraries resulted in the isolation of highly active chimeric variants. Of particular note, substitution of rGSTT2-2 helices 4 and 5 into the human enzyme framework resulted in a chimera comprised of 83% hGSTT1-1 amino acid sequence, but exhibiting high CMAC activity. Interestingly, this enzyme displayed moderate activity with ethacrynic acid (EA), a substrate not recognized by either parental protein.

The acquisition of promiscuous activities coupled with gene duplication has been suggested as a primary means by which nature evolves new enzyme functions.¹² A recent analysis detailed the role played by single mutations in the development of substrate selectivity and catalytic promiscuity.¹³ Directed evolution experiments have also yielded insights into how mutational enhancement of promiscuous activities may lead to new functions in the presence or absence of an associated selective pressure.^{14,15}

In this report, a flow cytometric screen for CMAC activity was employed in order to evaluate how different methods of sequence diversification impact the evolution of catalytic activity and substrate promiscuity in hGSTT1-1. Error-prone PCR, DNA shuffling, and saturation mutagenesis strategies were used to generate a variety of GST variants with enhanced CMAC activity. Detailed characterization of isolated enzyme variants resulted in the experimental identification of residues critical for activity. A basis for the rate enhancement observed in these variants is suggested by analysis of the recent hGSTT1-1 crystal structure.¹⁶ Kinetic analysis of isolated enzyme variants exhibiting a broad range of enhanced CMAC catalytic efficiencies revealed only a very weak correlation between the engineered CMAC activity and substrate promiscuity probed with three structurally diverse alternative electrophiles. In contrast, a previous study of homology-independent recombination between hGSTT1-1 and rGSTT2-2 led to the isolation of CMAC conjugating enzymes with very different promiscuous substrate activities, which, in the case of one electrophile, were found to correlate reasonably well with CMAC conjugating capacity.¹¹

RESULTS

Directed Evolution of CMAC Activity

An iterative approach was employed to evolve variants of hGSTT1-1 exhibiting high activity towards CMAC (Fig. 1). Initially, the *hGSTT1* gene (720 base pairs) was mutagenized by error-prone PCR, cloned downstream from the T7 promoter, and the DNA was electroporated into *Escherichia coli* (*E. coli*) BL21(DE3) yielding a library of $\sim 7.2 \times 10^5$ transformants (hereafter referred to as library HEP). Sequencing of four random clones indicated that the library contained an average of nine nucleotide substitutions per GSTT gene (1.25% error rate). The

transformant pool was grown to mid-log phase, and GSTT expression was induced by the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG). The induced cells were incubated with 10 μ M CMAC, and 5.8×10^6 cells were screened by flow cytometry to isolate highly fluorescent clones. The sorted cells were used to inoculate fresh media initiating a second round of growth, induction, and sorting. Iterative screening resulted in a progressive increase of the population's mean fluorescence (Fig. 2A). Following three rounds of screening, ten clones were selected at random, their whole cell fluorescence was determined individually by flow cytometry, and their respective GSTT genes were sequenced. Seven of the ten clones contained a Trp234-Arg mutation (Fig. 1 Supplemental), suggesting a dominant role for this residue in conferring high CMAC activity.

Approximately 700 clones isolated from the round three sort of library HEP were recombined and "backcrossed" with the parental *hGSTT1* gene by DNA family shuffling¹⁷ giving rise to a second library (library HEPSh) consisting of $\sim 7.8 \times 10^5$ transformants. Flow cytometric analysis revealed a bimodal population in which the lower 25% of events were centered on a mean fluorescence of 15, equivalent to the fluorescence of cells expressing the low activity hGSTT1-1 enzyme. This lower fluorescence population was eliminated after three rounds of sorting and growth of the isolated cells, as described above. The enriched population exhibited a mean whole-cell fluorescence similar to that conferred by the rGSTT2-2 enzyme (Fig. 2B). Thirty-six randomly selected clones from the HEPSh round three sort were grown individually, GSTT expression was induced with IPTG, and cellular CMAC fluorescence was determined by flow cytometry. GSTT genes from the seventeen most fluorescent clones were sequenced. Thirteen of the seventeen mutants encoded the Trp234-Arg substitution also observed in highly fluorescent HEP clones. Two other HEPSh clones encoded a Leu at residue 234. Other frequently mutated residues included: Ile32 to Met (4/17), Thr (2/17), Asn (2/17), or Val (1/17); and His176 to Gln (4/17).

To obtain clones with even higher CMAC activity, HEPSh7, the most fluorescent clone obtained following error-prone PCR and shuffling, was subjected to an additional round of error-prone mutagenesis. A library of 9.2×10^5 transformants with an average error rate of 0.33% was constructed (library HEPShEP), and high activity clones were enriched by four rounds of flow cytometric screening under increasingly stringent conditions achieved by inducing GST synthesis with progressively lower concentrations of IPTG (10 μ M, 5 μ M, 5 μ M, and 1 μ M, respectively) and incubation with decreasing concentrations of CMAC (1 μ M for round one and 0.5 μ M for subsequent rounds). CMAC activities of whole cell lysates were determined for 40 clones from the round four sort, and GSTT genes from the nine clones exhibiting the highest activities were sequenced. Notably, each gene was found to encode at least one mutation or insertion within the four C-terminal amino acids (Fig. 2 Supplemental).

The prevalence of mutations at Ile32, His176, and Trp234 following DNA shuffling suggested that these positions may be functionally important for high CMAC catalytic activity. In order to explore exhaustively the sequence space at positions 32, 176, and 234, the respective codons of the *hGSTT1* gene were subjected to combinatorial saturation mutagenesis by overlap extension PCR using degenerate primers, enabling evaluation of all possible amino acids at the targeted positions.¹⁸ The resulting library (S3; 1.6×10^6 transformants) represents a 50-fold coverage of the sequence space constituted by randomization of three codons via an NNS scheme. Sequencing of four randomly selected clones from the presort S3 population demonstrated diversity at the targeted residues, revealing little or no bias in the library as a result of the degenerate primers. The S3 library was screened by five rounds of sorting for CMAC activity using increasingly stringent conditions (IPTG = 100 μ M, 10 μ M, 1 μ M, 1 μ M, and 1 μ M, with CMAC = 5 μ M, 5 μ M, 5 μ M, 1 μ M, and 0.5 μ M, respectively). A total of twenty clones from the round 4 and 5 sorts were selected at random, and the respective CMAC activities of their whole-cell lysates were determined. GSTT genes of ten clones exhibiting the

highest initial CMAC reaction rates were sequenced. Seven of the ten clones encoded a consensus sequence at the targeted positions (Ile32Leu, His176Gln, and Trp234Thr, Table 1).

Enzyme Purification and Characterization

The hGSTT1-1 parental enzyme, variants isolated from the error-prone library (HEP clones), after shuffling (HEPSh clones), after a second round error-prone PCR (HEPShEP clones), and following saturation mutagenesis (S3 clones) were expressed as *N*-terminal His₆ tag fusions and purified to near homogeneity by IMAC chromatography. For comparison, the rat enzyme rGSTT2-2, which exhibits high CMAC activity,¹¹ was also expressed and purified. The enzymes' k_{cat} and K_M values for the conjugation of CMAC to GSH are given in Table 2.

Enzymes isolated from the first round of random mutagenesis and screening (HEP proteins) exhibited improved catalytic activity by at least one order of magnitude relative to the wild-type parent. The most efficient variant (HEP-C12 $k_{cat}/K_M = 1000 \text{ mM}^{-1} \text{ min}^{-1}$), exhibited a 320-fold rate enhancement ($k_{cat} = 29 \text{ min}^{-1}$) compared to hGSTT1-1 ($k_{cat} = 0.09 \text{ min}^{-1}$). However, the HEP-C12 protein was found to have compromised stability in solution, as evidenced by its propensity to precipitate even at moderate concentrations (<1mg/ml).

Highly fluorescent clones isolated following DNA shuffling were ranked-ordered with respect to CMAC activity in microtiter well plates. The four enzymes that conferred the highest initial CMAC reaction rates in whole-cell lysates (HEPSh7, HEPSh9, HEPSh14, and HEPSh15) were purified and characterized. The CMAC K_M values of the four enzymes were comparable. All four variants were found to catalyze the conjugation of CMAC and GSH with k_{cat} values similar to, or greater than, HEP-C12, resulting in catalytic efficiencies that varied between 700-3,400 $\text{mM}^{-1} \text{ min}^{-1}$. The most active enzyme, HEPSh7, was >2,000-fold more efficient with CMAC compared to the parental hGSTT1-1 ($k_{cat}/K_M = 3,400 \text{ mM}^{-1} \text{ min}^{-1}$ and $1.6 \text{ mM}^{-1} \text{ min}^{-1}$, respectively). It is noteworthy that four of the five most active HEP and HEPSh enzymes encoded Arg at position 234 (Fig. 3 Supplemental). The remaining variant (HEPSh9) possessed Leu at 234, and exhibited a 3.5 to 5.5-fold lower catalytic efficiency with GSH ($k_{cat}/K_M = 6.9 \text{ mM}^{-1} \text{ min}^{-1}$).

The catalytic efficiency of HEPSh7 was improved further by an additional round of error-prone PCR and screening. Two of the variant enzymes isolated from the resulting library (HEPShEP) possessed significantly decreased K_M values for CMAC (Table 2). The isolation of enzymes with decreased K_M values is consistent with the increased stringency of the screening conditions that employed progressively lower concentrations of the CMAC substrate. The best enzyme (HEPShEP-aa7) retained high catalytic activity while exhibiting a substantially reduced K_M of 2.4 μM , resulting in an overall 20,000-fold improvement in catalytic efficiency relative to the hGSTT1-1 human parent.

Combinatorial saturation mutagenesis of positions 34, 176, and 234 led to the isolation of variants S3-1 (Ile32-Ser; His176-Gln; Trp234-Thr) and S3-7 (Ile32-Leu; His176-Gln; Trp234-Thr). These two enzymes possessed k_{cat} values of approximately 86 min^{-1} , equivalent to that displayed by HEPSh7, the most active enzyme obtained by directed evolution. However, S3-1 and S3-7 exhibited lower K_M values for CMAC, resulting in modest increases in catalytic efficiency relative to HEPSh7, but still significantly less than that of HEPShEP-aa7.

The evolution of substrate promiscuity was evaluated with three structurally distinct electrophiles (Fig. 3). In analogy to other studies,¹⁵ increasing activity towards the CMAC selection substrate was generally accompanied by higher activity with one or more of the alternative electrophiles. However, little or no correlation was observed between activity with the selection substrate and any given alternate substrate (Fig. 4 Supplemental). The most pronounced influence on substrate promiscuity was seen with phenethyl isothiocyanate

(PEITC). Relative to hGSTT1-1, the average PEITC rate enhancement was approximately 20-fold, and tended to increase with CMAC activity, although not in a well defined manner. The average enhancement of 1-chloro-2,4-dinitrobenzene (CDNB) activity was less marked, with the exception of HEPShEP-aa7 that displayed a 100-fold increase. Cross-reactivity with EA was the least pronounced of the three alternative electrophiles tested.

Site saturation mutagenesis of Trp234

The high frequency of Trp234 mutation to Arg during directed evolution of CMAC activity demonstrated the importance of this residue for efficient catalysis with the selection substrate, and was consistent with recent observations regarding the effect of the Trp234Arg mutation on hGSTT1-1 activity.⁸ However, the results of combinatorial saturation mutagenesis at positions 32, 176 and 234 showed that Arg was not the preferred 234 residue. This observation led to a detailed examination of the functional significance of hGSTT1-1 residue 234 using site-saturation mutagenesis. Nineteen mutants encoding all possible amino acid substitutions at residue 234 were constructed individually, expressed in bacteria, and the CMAC activities of crude cell lysates were determined and normalized to expression level. Analysis of the relative catalytic activities revealed two startling findings. First, every other amino acid exhibited improved activity with CMAC compared to the wild-type Trp234 residue (Fig. 4). Second, Trp234Arg, the predominant substitution found in clones obtained by directed evolution, was only the sixth best point mutant. The five clones showing the highest levels of catalytic activity in crude lysates were Trp234Thr, Trp234Pro, Trp234Ser, Trp234Asn, and Trp234Gln. Three mutants (Trp234Thr, Trp234Pro, and Trp234Arg) were purified, and their CMAC kinetics determined (Table 2). The catalytic rates and CMAC efficiencies corresponded closely with the relative activities observed in crude lysates.

DISCUSSION

The broad spectrum of xenobiotics targeted by GSTs renders these enzymes particularly interesting model systems for evaluating the effect of mutations on catalytic activity and substrate specificity. However, the dearth of truly high-throughput quantitative screens for GST activity has required most studies to either focus on the role played by single amino acid residues,^{8,19} or employ small libraries that could be interrogated with microtiter well plate assays.^{20,21} Here, a single-cell, quantitative, fluorescent assay was employed to screen a series of large GST libraries, typically comprised of 700,000-1,000,000 clones each, leading to the isolation of enzymes exhibiting up to a 2×10^4 increase in k_{cat}/K_M . Detailed kinetic analysis of clones isolated from successive libraries provided information on how the evolution of high activity for the selection substrate affects substrate promiscuity.

An iterative directed evolution approach alternating random mutagenesis and DNA shuffling was employed to engineer highly active CMAC conjugating enzymes. After each round of sequence diversification, flow cytometry was exploited to isolate clones with the greatest single cell fluorescence resulting from the conjugation of CMAC to GSH. Interestingly, a Trp234Arg mutation became dominant during the first round of mutagenesis, and was retained by the majority of selected clones from the second and third generation libraries.

While this work was in progress, Shokeer *et al.* also reported that mutation of hGSTT1-1 Trp234 to Arg results in enhanced activity with various electrophiles.⁸ To examine further the significance of residue 234 in hGSTT1-1 activity, we constructed all 19 point mutants at this position and analyzed the relative catalytic activities of their crude lysates towards CMAC. Surprisingly, all of the 19 other amino acids at position 234 showed higher activity than the hGSTT1-1 wild-type Trp234. Detailed kinetic analysis of three purified point mutants (Trp234Arg, Trp234Pro, and Trp234Thr; Table 2) yielded results that closely parallel the cell lysate assays. Because the 234 residue extends into the substrate binding pocket, it has been

proposed that the large size of the wild-type Trp234 presents a steric barrier to binding of both GSH and electrophilic substrates. Thus, all of the other 19 amino acids likely facilitate CMAC-GSH conjugation by increasing the active site free volume, in analogy to the recent Trp234Arg hGSTT1-1 structure.¹⁶

Of particular interest was the fact that substitution of Thr, Pro, Ser, Asn, or Gln at position 234 resulted in higher relative CMAC activity than the Trp234Arg mutation that so frequently appeared in selected clones (Fig. 4 and Table 2). Significantly, five of the six amino acid substitutions that result in the highest activity (including Trp234Arg) have side chains capable of donating a hydrogen bond. In the recent structural analysis of the Trp234Arg hGSTT1-1 variant, a salt bridge was seen between the Trp234Arg guanidinium and the GSH glycine carboxylate.¹⁶ Therefore, it seems reasonable to propose that Trp234Thr, Ser, Asn, and Gln may be facilitating catalysis in part by forming a hydrogen bond with the GSH glycine carboxylate group. However, the GSH K_M of Trp234Thr (the most active 234 point mutant) is approximately an order of magnitude larger than that of the Trp234Arg variant (Table 2). Thus, the exact mechanism by which hydrogen bonding between GSH and residue 234 might influence catalysis remains unclear. Nevertheless, there appears to be a strong selective pressure for a hydrogen bond or salt bridge donor at this position, both in the context of the current study and in natural evolution of non-human mammalian theta class GSTs (Fig. 5 Supplemental).

In contrast to the other catalytically proficient Trp234 point mutants, the Trp234Pro side chain cannot participate in hydrogen bonding. However, it is likely that proline at position 234 disrupts the $\alpha 9$ helix, resulting in a conformational re-organization of the C-terminal region in a manner favorable to catalysis.

Assuming a maximum of one base substitution per codon during error-prone PCR, the TGG Trp234 codon (sense strand) of the parental gene can theoretically be mutated to encode five alternate amino acids and two stop codons. However, considering that only certain nucleotide substitutions are observed at significant frequencies during error-prone PCR mutagenesis with Taq polymerase,²² the most likely mutations at Trp234 yield either Arg (CGG and AGG) or stop codons (TAG and TGA). The two Trp234Leu clones isolated from the HEPSh library apparently resulted from a much less common G to T or C to A transversion. Thus, absence of the highly active Trp234Thr, Trp234Ser, Trp234Pro, Trp234Asn, and Trp234Gln substitutions in the enriched error-prone and shuffled libraries is most likely a consequence of the error-prone PCR mutagenesis strategy used for genetic diversification. These results underscore the importance of using saturation mutagenesis strategies at key residues to overcome the codon substitution limitations imposed by error-prone PCR and shuffling strategies.^{18,23}

In addition to Trp234Arg, enzymes isolated after DNA shuffling often contained mutations at residues Ile32 (53%) and His176 (24%). The frequency of mutation at these three positions suggests a high degree of plasticity that allows modulation of catalytic activity. To examine the impact of these residues in finer detail, combinatorial saturation mutagenesis was performed at codons 32, 176, and 234. Five rounds of flow cytometric screening for CMAC activity led to a consensus sequence in which position 176 was fixed to Gln, position 234 was occupied primarily by Thr (8 of 10 clones), and position 32 by Leu (7 of 10 clones) (Table 1).

The Gln176 residue aligns structurally with the highly conserved Gln175 of natural T2-2 GSTs,¹⁶ and may play a role in transition state stabilization (see below). Examining the hGSTT1-1 crystal structure showed that residue 32 is solvent exposed and located in β -strand 2, more than 10 Å from the active site. Thus, it is difficult to draw conclusions regarding this residue's influence on activity, particularly given the conservative nature of the Ile32Leu substitution. Finally, Thr was preferred at residue 234, a result consistent with the site saturation studies of

this position (Fig. 4). Thr likely provides a hydrogen bond donating group while reducing steric bulk in the active site, as discussed above.

Evolutionary enhancement of promiscuous activities is thought to play a substantial role in the creation of new enzyme functions.^{12,24} Recent experiments have also shown that engineered enzymes often develop promiscuous activities during the directed evolution process.^{15,25} Similarly, enzymes selected here for enhanced conjugation of GSH and CMAC following error-prone PCR, family shuffling, or saturation mutagenesis of hGSTT1-1 typically acquired some level of promiscuous substrate activity. In general, the variants exhibited marginal increases in specific activity with CDNB and EA, and more substantial enhancements towards PEITC. However, substrate promiscuity did not correlate well with catalytic proficiency towards the CMAC selection substrate. In other words, it appears that mutations beneficial for conjugation of the selection substrate give rise to promiscuous cross-reactivities in a largely stochastic manner.

A closer inspection of the four enzymes with PEITC activity in excess of 2 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ (HEPSh7, HEPSh9, S3-7, and S3-1) suggests that His176Gln (common to each of the four variants) plays a role in recognition of this substrate. Modeling predicts Gln176 to be in close proximity to the putative iodide leaving group of a bound 1-iodohexane-glutathione conjugate (Fig. 5). As a result, this mutation may lower transition state energy via hydrogen bonding to the growing negative charge of the leaving group resulting from GSH nucleophilic attack. It is reasonable to predict that, similar to 1-iodohexane and CMAC, the reaction of GSH with PEITC also results in a transition state with concentrated negative charge immediately adjacent to the electrophilic carbon. Thus, high level activity with both CMAC and PEITC may derive from electronic similarities in their transition states. In contrast, both CDNB and ethacrynic acid are expected to develop significantly more diffuse and spatially distant negative charge during reaction with GSH, minimizing any stabilizing effect of His176-Gln.

Although enzymes from this study showed a general preference for cross-reactivity with PEITC, a radar plot of specific activities (CMAC, CDNB and PEITC) averaged among characterized members of each library revealed diverging cross-reactivity for the third generation HEPShEP variants (Fig. 6). The third generation of evolved enzymes exhibited a decrease in activity with PEITC relative to their HEPSh predecessors, but a notable increase in activity with CDNB. Thus, random mutagenesis and shuffling led to highly active CMAC catalysts with cross-reactivity against either PEITC or CDNB, but only following the accumulation of mutations from more than one round of genetic diversification. In contrast, combinatorial saturation mutagenesis (library S3) yielded highly active CMAC conjugating enzymes in a single round, but limited cross-reactivity predominantly to the PEITC substrate.

Striking differences were noted with respect to promiscuous activities of enzymes isolated in these studies compared to those of enzymes previously obtained by homology-independent recombination of hGSTT1-1 and rGSTT2-2.¹¹ In contrast to the *hGSTT1* mutants described above, chimeras generated by homology-independent recombination in the previous study exhibited very little increase in activity with PEITC. Furthermore, variants from the current study showed only modest increases with CDNB, while the homology-independent chimeras developed remarkable levels of activity with this electrophile (SCR23 being the single exception). Additionally, the CMAC and CDNB activities of the chimeric enzymes were correlated to a significant degree (Fig. 4 Supplemental). The precise reason(s) for this correlation remains unclear, but it may be that under selective pressure for high CMAC activity, chimeras of the rat and human proteins acquire relevant rGSTT2-2 catalytic residues that may also play a role in activity with CDNB, which is a good substrate for the rat enzyme.

Alternatively, CDNB specifying residues of rGSTT2-2 may “hitchhike” with CMAC determinants if the two share close proximity in the primary amino acid sequence.

The chimeric enzyme possessing the highest level of activity with CMAC (SCR23) exhibited a substrate promiscuity profile entirely unique among all of the GSTT enzymes examined in either study. This enzyme was the only CMAC conjugating catalyst to exhibit appreciable activity with EA. The distinct substrate selectivity of SCR23 demonstrates the capacity of homology-independent recombination to sample regions of functional sequence space not readily accessible by other diversification strategies. For comparison, SCR23 possessed a CMAC $k_{cat} = 27 \text{ min}^{-1}$, approximately 1/3 of that seen with the best clones described in this study.

CONCLUSIONS

This report details the *in vitro* evolution of catalytic activity in the human theta class glutathione transferase hGSTT1-1. Iterative mutagenesis coupled with flow cytometric screening of large libraries yielded an array of enzymes exhibiting as much as 2×10^4 greater catalytic efficiency than the human parental protein. The high activity enzymes invariably contained mutations of residue Trp234. Because of codon constraints imposed by random mutagenesis using error-prone PCR with Taq polymerase, Arg is the only alternative amino acid that can be readily accessed at residue 234, and was found in the majority of the clones analyzed here. However, site saturation mutagenesis of codon 234 revealed that all of the 19 other amino acids are better than the wild-type Trp residue with respect to CMAC catalysis. In particular, Trp234Thr conferred the highest level of catalytic activity, with Trp234Arg being only the sixth best amino acid substitution. Therefore, it is no surprise that most highly active enzymes isolated from a combinatorial saturation mutagenesis library of residues 32, 176 and 234 encoded Thr at residue 234.

The acquisition of substrate promiscuity during evolution of CMAC activity was found to be dependent on library construction methodology. In the present study, a general trend of greater promiscuity with at least one of the alternative electrophiles was seen in clones exhibiting enhanced activity with the CMAC selection substrate. The engineered hGSTT1-1 variants typically showed a preference for cross-reactivity with PEITC, although HEPShEP-aa7, the most efficient enzyme isolated, exhibited greater activity with CDNB. However, CMAC activity did not correlate significantly with any of the alternant substrate activities. The previous homology-independent recombination libraries yielded chimeric enzymes that showed noticeably different patterns of promiscuity with the alternative substrates.¹¹ For example, the chimeras exhibited little reactivity with PEITC, but showed a reasonable correlation between activity with the CMAC selection substrate and the alternative substrate CDNB. Thus, disparate sequence diversification strategies provide parallel routes to the directed evolution of a non-natural activity in hGSTT1-1, but can result in orthogonal substrate promiscuity profiles for the corresponding variants.

EXPERIMENTAL

Reagents

Oligonucleotides (50nmol scale, desalted) were purchased from Integrated DNA Technologies (Coralville, IA). pET-28a plasmid was obtained from Novagen (San Diego, CA). Restriction enzymes, modification enzymes, Vent polymerase, bovine serum albumin (BSA), and dNTPs were obtained from New England Biolabs Inc. (Beverly, MA). Taq Polymerase was obtained from Invitrogen (Carlsbad, CA). QIAquick® Gel extraction, QIAprep® Spin plasmid purification, and QIAquick® PCR purification kits were purchased from QIAGEN (Valencia, CA). All growth media were obtained from Difco Ltd. (UK). CellTracker Blue™ 7-amino-4-

chloromethyl coumarin was purchased from Invitrogen/Molecular Probes (Eugene, OR). Unless specifically noted, all other reagents were from Sigma-Aldrich Co. Ltd or Fluka. Sequencing was performed using a dideoxy terminator protocol.

Library Construction

The error-prone hGSTT1-1 library HEP was generated using the authentic hGSTT1-1 gene, cloned into the NcoI and BamHI sites of pET-28a, as a template and the T7 promoter and T7 terminator primers. Reaction solutions (100 μ l each) contained 0.23 mM dATP, 0.20 mM dCTP, 0.57 mM dGTP, 4.0 mM dTTP, 0.5 mM MnCl₂, 5.2 mM MgCl₂, 3 fM template, 1 μ M each primer, 10 mM Tris (pH 8.3), 50 mM KCl, 5ng BSA, and 5 units of Taq polymerase. Reaction profiles were: 95°C, 2 min; 16 cycles – 94°C, 1 min / 51°C, 1 min / 72°C, 3 min; 72°C, 7 min. Product was purified using a QIAGEN PCR purification kit. The purified DNA was used as template in an additional error-prone PCR reaction as described above resulting in a second error-prone gene library. Both gene libraries were combined and digested with NcoI and BamHI. Digested product was purified on a 1% agarose gel (QIAGEN extracted), and quantitated. This insert was ligated in a 3:1 molar ratio with similarly digested and dephosphorylated pET-28a vector (8.5 μ g of total DNA) at 25°C overnight, and the solution was heat inactivated and desalted on a nitrocellulose membrane. The ligation product was ethanol precipitated and re-dissolved in 15 μ l of ddH₂O. This solution was divided into three 5 μ l portions and transformed by electroporation into Jude-1 cells (DH10B harboring the F' factor derived from XL1-blue, A. Hayhurst unpublished data) resulting in $\sim 7.2 \times 10^5$ independent clones. Plasmid DNA was isolated from this library and re-transformed into *E. coli* BL21(DE3) (F⁻ *ompT hsdS_B(r_B⁻ m_B⁻) gal dcm* (DE3)) prior to screening for CMAC activity.

For the construction of the second generation library HEPSh, genes isolated from the HEP round 3 sort were amplified by PCR with the T7 promoter and T7 terminator primers, and product was purified with a QIAGEN PCR purification kit. These genes (2 μ g total) were digested in 50 μ l of 50 mM Tris, 1 mM MgCl₂, pH 7.4 with 0.2 units of DNaseI at 25°C for 30 minutes. A 2 μ g sample of the authentic hGSTT1-1 gene was digested independently in an identical manner. Digestion reactions were heat inactivated at 90°C for 10 minutes, and agarose gel electrophoresis confirmed the fragments to be less than 100 base pairs in length. The round 3 error-prone fragments (722 ng) were mixed with authentic hGSTT1-1 fragments (44 ng), and the mixture was combined with 100 μ M each dNTP, 1.9 units of Vent polymerase, and 0.6 units of Taq polymerase in 50 μ l of 1x ThermolPol PCR buffer. The gene fragments were reassembled using the following reaction profile: 94°C, 1 min; 50 cycles – 94°C, 30 sec / 50°C, 30 sec / 72°C, 30 sec; 72°C, 7 min. Analysis of the reassembly product by agarose gel electrophoresis revealed a smear encompassing the 950 bp target length. Five μ l of the assembly reaction mixture was used as template in a 100 μ l PCR amplification with the T7 promoter and T7 terminator primers (using the same 3:1 Vent:Taq polymerase mixture). Agarose gel electrophoresis revealed one prominent product band of the expected size. This product was QIAGEN PCR purified, digested with NcoI and BamHI, cloned into pET-28a, and transformed into *E. coli* as described above resulting in $\sim 7.8 \times 10^5$ independent clones.

The third generation library HEPShEP was constructed by error-prone PCR essentially as described above. Plasmid purified from clone HEPSh7 was used as template, and the targeted error rate was 0.5%, i.e. reaction solutions (100 μ l each) contained 0.23 mM dATP, 0.20 mM dCTP, 0.57 mM dGTP, 4.0 mM dTTP, 0.5 mM MnCl₂, 5.2 mM MgCl₂, 3 fM template, 1 μ M each primer, 10 mM Tris (pH 8.3), 50 mM KCl, 5ng BSA, and 5 units of Taq polymerase. The gene library was cloned and transformed into *E. coli* as described above yielding $\sim 9.2 \times 10^5$ independent clones.

The site saturation library S3 was constructed in a three-part overlap extension PCR reaction using the PAGE purified degenerate primers 5'hGST32-RND (5'-NNSGTGGATCTGATTAAAGGTCAGC-3'), 3'hGST32-RND (5'-GCTGACCTTTAATCAGATCCACSNNGCGCAGCTCGAAGGGAATGTCG-3'), 5'hGST176-RND (5'-NNSCCCGTGGGTGCTGGCTGCC-3'), 3'hGST176-RND (5'-GGCAGCCAGCACCCACGGGSNNCATCAGCTCCGTGATGGC-3'), and hGST234-RND (5'-AGCGGGATCCTTAACGGATCATGGCCAGCACSNNGGCATCAGCTTCTGCTTTATGG-3'). The purified gene products were cloned and transformed as described above yielding $\sim 1.6 \times 10^6$ independent clones.

Site directed mutants of residue 234 were constructed independently using 19 mutagenic 3'-primers of the form 5'-

CGCGGATCCTTATTAACGGATCATGGCCAGCACXXXGG-3' (BamHI cloning site in bold, dual stop codons underlined, and XXX representing codon 234).

Flow Cytometric Screening

Flow cytometric screening of HEP and HEPSh for CMAC activity was carried out essentially as previously described.¹¹ Briefly, cells were grown overnight at 30°C in LB media supplemented with 50 µg/ml kanamycin (LB/Kan), subcultured into fresh media (1:100 dilution), and grown at 37°C with vigorous shaking. When the absorbance (OD₆₀₀) reached 0.5–0.7, the cells were transferred to a 25°C shaker and allowed to equilibrate for 30 minutes. Subsequently, IPTG was added to a final concentration of 1 mM, and the cultures were incubated for an additional 3–4 hours prior to harvesting. Aliquots (1 ml) were placed in a centrifuge at 2040xg for 5 minutes, the cell pellet was resuspended in 1 ml of phosphate buffered saline (PBS, pH 7.4), and the sample was placed on ice. The cells were diluted with PBS as needed to obtain a flow cytometric event rate of 5–15,000 per second. A stock solution of 10 mM CMAC (Molecular Probes – Eugene, OR) in dimethylformamide was then added to a final concentration of 10 µM. The final solution was kept at 25°C for 30 seconds prior to sorting, and sorts were terminated 2 minutes after addition of the CMAC substrate.

Sorting of HEPShEP required minor modifications. Specifically, the isopropyl-β-D-thiogalactoside (IPTG) inducer concentration employed in the round 1 sort was 10 µM. This IPTG concentration was progressively decreased from one round to the next reaching a final concentration of 1 µM in the round 4 sort. Likewise, the round 1 sort was carried out with 1 µM CMAC substrate. This concentration was further reduced to 500 nM for rounds 2, 3, and 4. Sorting of library S3 employed a similar strategy of increasing stringency from round to round. Namely, IPTG inducer concentrations were progressively decreased from 100 µM in round 1 to 1 µM in round 5. Likewise, the concentration of CMAC was progressively decreased from 5 µM to 500 nM.

Determination of CMAC Activity in Whole Cell Lysates

Monoclonal cultures were grown and induced with 1mM IPTG as described above. Aliquots (1.5 ml) were pelleted by centrifugation, and the pellets were resuspended in 1 ml of 10 mM Tris, 500 mM NaCl, 10% glycerol, pH 8.0. These suspensions were lysed via a single pass through a French pressure cell, and the lysates were centrifuged at 16,100xg for 45 minutes at 4°C. The supernatant was removed and placed on ice. Aliquots (15 µl) were analyzed by SDS-PAGE, and relative GSTT expression levels were determined by Coomassie staining. The CMAC activity of each lysate was determined as described previously using 100 µM CMAC and 10 mM GSH.¹¹ Initial rates were estimated from the linear portions of fluorescence vs. time curves. These rates were normalized to expression levels, and the most active enzymes were selected for purification and detailed kinetic analysis.

Enzyme Purification and Kinetic Analysis

Enzymes of interest were fused to *N*-terminal His₆ tags, purified by IMAC chromatography, and their kinetics with CMAC, PEITC, CDNB, and EA were determined as previously described.¹¹

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GST	glutathione transferase
GSH	glutathione
hGSTT1-1	human glutathione transferase theta 1-1
rGSTT2-2	rat glutathione transferase theta 2-2
CMAC	7-amino-4-chloromethyl coumarin
EA	ethacrynic acid
CDNB	chloro-2,4-dinitrobenzene
PEITC	phenethyl isothiocyanate
E. coli	Escherichia coli

HEP	1 st generation error-prone library
IPTG	isopropyl-beta-D-thiogalactopyranoside
HEPSh	2 nd generation shuffled library
S3	saturation library of codons 32, 176 and 234 HEPShEP, 3 rd generation error-prone library

Figure 1. Origins and fitness of selected CMAC enzymes. k_{cat}/K_M values for the enzymes shown are provided in Table 2. The construction and screening of the libraries is discussed in the Materials and Methods section.

Figure 2.

Whole-cell fluorescence histograms of GSTT expressing *E. coli* incubated with CMAC. Red: Cells expressing hGSTT1-1; Blue: rGSTT2-2 expressing cells. (A) Fluorescence of cells expressing the HEP library prior to sorting (0), cells collected after 1 round of sorting (I), after 2 rounds (II), and after 3 rounds (III) shown in pink. B) Fluorescence of cells expressing the HEPSh library prior to sorting (0), after 1 round (I), and after 2 rounds (II) of sorting shown in purple. Cells collected after round 3 did not show a further increase in mean fluorescence signal (data not shown).

Figure 3.

A) Mean GSTT specific activities with EA (yellow), CDNB (green), PEITC (orange), and CMAC (blue). The rGSTT2-2 specific activity for CDNB is $13 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (not shown). Standard deviations were typically between ± 1 and 20% of the observed value for specific activities larger than 0.01. B) Structures of electrophilic substrates employed in these studies. Site of nucleophilic attack by GSH thiolate indicated with arrows.

Figure 4. Relative CMAC activity of Trp234 point mutants. Initial rates measured with crude cell lysates and normalized to expression levels as determined by SDS-PAGE gel electrophoresis. Variants denoted by the one letter amino acid code of residue 234.

Figure 5.

View of GSTT monomeric subunit active site based on hGSTT1-1 Trp234Arg crystal structure (PDB ID 2C3Q). The three frequently mutated positions Trp234Arg (green), His176Gln (pink), and Ile32 (light blue) are represented as molecular surfaces. The GSH conjugate to n-hexane (yellow) is bound in the active site. The putative iodide leaving group resulting from reaction of GSH with 1-iodohexane is shown as an orange ball. Note the close proximity of the Gln176 side chain to this iodide atom.

Figure 6.

Radar plot of specific activities from figure 3 ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) averaged by library. (—, black) hGSTT1-1 parent, (—, red) library HEP clones, (—, blue) library HEPSh clones, (—, purple) library HEPShEP clones, (—, green) library S3 clones. Figure S1 - Amino acid alignment of 10 error-prone GSTs selected from sort round 3. Parental human sequence depicted at top, and consensus sequence at bottom. Positions where all residues are identical are shown as black text on white. Positions where one or more sequences are mutated have residues matching the consensus in black on grey and those differing from the consensus depicted as white on black. Note the consensus Trp234Arg mutation.

Table 1

Mutations in high activity saturation clones (library S3)

Enzyme	Residue			Other Mutations
	32	176	234	
hGSTT1-1	Ile	His	Trp	N/A
S3-1	Ser	Gln	Thr	N/A
S3-4	His	Gln	Arg	M238V
S3-5	Pro	Gln	Pro	N/A
S3-9	Leu	Gln	Thr	D25E
S3-7*	Leu	Gln	Thr	N/A

* Consensus sequence encoded by 60% of the sequenced clones

Table 2
Kinetic parameters of evolved GST enzymes for the conjugation of CMAC with GSH

Enzyme	K_M (CMAC) (μM)	K_M (GSH) (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (CMAC) ($\text{mM}^{-1}\text{min}^{-1}$)	k_{cat}/K_M (GSH) ($\text{mM}^{-1}\text{min}^{-1}$)
hGSTT1-1	54 $\pm$ 5	6400 $\pm$ 700	0.09 $\pm$ 0.01	1.6 $\pm$ 0.3	0.014 $\pm$ 0.003
rGSTT2-2	11 $\pm$ 1	420 $\pm$ 70	7 $\pm$ 1	700 $\pm$ 200	17 $\pm$ 7
HEP-B1	89 $\pm$ 3	2300 $\pm$ 300	1.3 $\pm$ 0.1	14 $\pm$ 2	0.5 $\pm$ 0.1
HEP-B10	68 $\pm$ 6	2900 $\pm$ 400	4.0 $\pm$ 0.3	60 $\pm$ 10	1.4 $\pm$ 0.3
HEP-C1	51 $\pm$ 7	3000 $\pm$ 400	2.3 $\pm$ 0.1	50 $\pm$ 10	0.8 $\pm$ 0.2
HEP-C12	30 $\pm$ 4	1160 $\pm$ 40	29 $\pm$ 2	1000 $\pm$ 200	25 $\pm$ 3
HEPSb7	24 $\pm$ 2	2100 $\pm$ 200	81.7 $\pm$ 0.3	3400 $\pm$ 300	39 $\pm$ 4
HEPSb9	37 $\pm$ 4	3700 $\pm$ 300	26.0 $\pm$ 0.4	700 $\pm$ 100	6.9 $\pm$ 0.7
HEPSb14	34 $\pm$ 4	1160 $\pm$ 40	28 $\pm$ 3	800 $\pm$ 200	24 $\pm$ 4
HEPSb15	29 $\pm$ 3	1200 $\pm$ 100	41 $\pm$ 2	1400 $\pm$ 300	34 $\pm$ 5
HEPSbEP-A7	15 $\pm$ 2	7000 $\pm$ 1000	50 $\pm$ 6	3300 $\pm$ 800	7.1 $\pm$ 0.8
HEPSbEP-aa7	2.4 $\pm$ 0.2	ND*	76 $\pm$ 2	32000 $\pm$ 5000	ND*
S3-1	18.6 $\pm$ 0.3	5200 $\pm$ 10	86 $\pm$ 3	4600 $\pm$ 100	16.6 $\pm$ 0.5
S3-7	14 $\pm$ 1	4600 $\pm$ 100	86 $\pm$ 9	6000 $\pm$ 700	19 $\pm$ 2
hT1-1 W234R	49 $\pm$ 8	383 $\pm$ 40	7.5 $\pm$ 0.8	160 $\pm$ 40	19 $\pm$ 4
hT1-1 W234P	50 $\pm$ 10	3400 $\pm$ 700	9 $\pm$ 1	200 $\pm$ 100	3.1 $\pm$ 0.7
hT1-1 W234T	37 $\pm$ 4	3320 $\pm$ 20	17 $\pm$ 1	400 $\pm$ 100	5.6 $\pm$ 0.3

* HEPSbEP-aa7 exhibited non-Michaelis-Menton kinetics with respect to the GSH Substrate