Alternative mutations of a positively selected residue elicit gain or loss of functionalities in enzyme evolution

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All molecular species in an organism are connected physically and functionally to other molecules. In evolving systems, it is not obvious to what extent functional properties of a protein can change to selective advantage and leave intact favorable traits previously acquired. This uncertainty has particular significance in the evolution of novel pathways for detoxication, because an organism challenged with new xenobiotics in the environment may still require biotransformation of previously encountered toxins. Positive selection has been proposed as an evolutionary mechanism for facile adaptive responses of proteins to changing conditions. Here, we show, by saturation mutagenesis, that mutations of a hypervariable residue in human glutathione transferase M2-2 can differentially change the enzyme's substrate-activity profile with alternative substrates and, furthermore, enable or disable dissimilar chemical reactions. Crystal structures demonstrate that activity with epoxides is enabled through removal of steric hindrance from a methyl group, whereas activities with an orthoquinone and a nitroso donor are maintained in the variant enzymes. Given the diversity of cellular activities in which a single protein can be engaged, the selective transmutation of functional properties has general significance in molecular evolution.

enabling alternative reactions | glutathione transferase | GST | positive selection | saturation mutagenesis

The evolution of protein functions is a multiparameter optimization that is constrained by boundary conditions set by ambient physical conditions, interactions with other molecules, and possible alternative functions. Expressivity of the cognate gene in the ribosomal translation system and solubility and stability of the protein in the cellular milieu are obvious restrictions. Furthermore, it is estimated that >50% of the human genes undergo alternative splicing (1), and the change in the coding sequence for a protein may jeopardize the function of an alternatively spliced mRNA. These and other limitations attenuate mutational changes that otherwise would be feasible and, presumably, partly explain why molecular structures in biological systems are highly conserved. This study shows how point mutations in a single positively selected position can lead to drastic changes of both substrate selectivities and physical properties of an enzyme and indicates how a protein could rapidly respond in multiple dimensions of functional space, optionally with minimal effects on alternative functions of the protein.

GSTs are members of a diverse superfamily of detoxication enzymes. The GSTs have evolved through divergent evolution from a common ancestor, and the enzymes are divided into classes based on similarities in the primary structure (2–4). As detoxication enzymes, GSTs are endowed with broad substrate specificities and functional plasticity. These qualities are valuable in providing the organism with protection against various harmful chemical species but may also be important parameters contributing to the diversification of the gene family and the evolution of new enzymatic functions. The Mu class is a lineage in the evolution of the GST gene superfamily with substantial potential for novel functions and is currently one of the largest mammalian GST classes, consisting of five structural genes in humans and at least six in mice and rats (3).

In a previous study, we used evolutionary rate analysis to identify a number of hypervariable positions in the Mu-class sequences (5); such positions are under positive selection for change and possibly contribute to functional variations among the divergent paralogues. The hypervariable residue 210 differs between human GST M1-1 and GST M2-2, two enzymes that display distinct differences in substrate-activity profiles. Thus, it appears that residue 210 could serve as a powerful modulator of catalytic activity in Mu-class GSTs. In this investigation, the evolutionary potency of this modulating function was addressed by saturation mutagenesis of position 210 in GST M2-2. The properties of the 20 different enzyme variants, denoted M2Ala, M2Gly, and so on, were clearly divergent, in accordance with the paradigm of positive selection.

Results

Enabling and Disabling of Diverse Alternative Activities. GSTs catalyze distinct types of chemical reactions including substitutions, additions, and isomerizations. Mu-class GSTs have prominent activities in the first two categories. The effects of mutations of residue 210 on the substitution reactions were assayed with the general GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) and the GST M2-2 distinctive substrate 2-cyano-1,3-dimethyl-1-nitrosoguanidine (cyanoDMNG). The influence on addition reactions was investigated by using an alternative GST M2-2 substrate, aminochrome, and three epoxides with different molecular sizes, the largest being *trans*-stilbene oxide (tSBO), followed by (2*S*,3*S*)-(+)-3-(4-nitrophenyl)glycidol (NPG), and styrene-7,8-oxide (SO).

All enzyme variants with mutations in residue 210 displayed activity toward CDNB, cyanoDMNG, and aminochrome but with different substrate-selectivity profiles (Fig. 1*A*; and see Table 1, which is published as supporting information on the PNAS web site). Least favorable to high activities with these substrates were mutants with aromatic or basic amino acids, such as M2Trp and M2Arg, whereas M2Gly, M2Ser, and M2Thr had high activities with all three substrates. Surprisingly, M2Pro had high activity with aminochrome. Six of the variant GST M2-2 enzymes (M2Gly, M2Ala, M2Leu, M2Val, M2Ser, and M2Thr) were further characterized by steady-state kinetics by using

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; cyanoDMNG, 2-cyano-1,3-dimethyl-1-nitrosoguanidine; NPG, (2*S*,3*S*)-(+)-3-(4-nitrophenyl)glycidol; SO, styrene-7,8-oxide; tSBO, *trans*-stilbene oxide.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2C4J).

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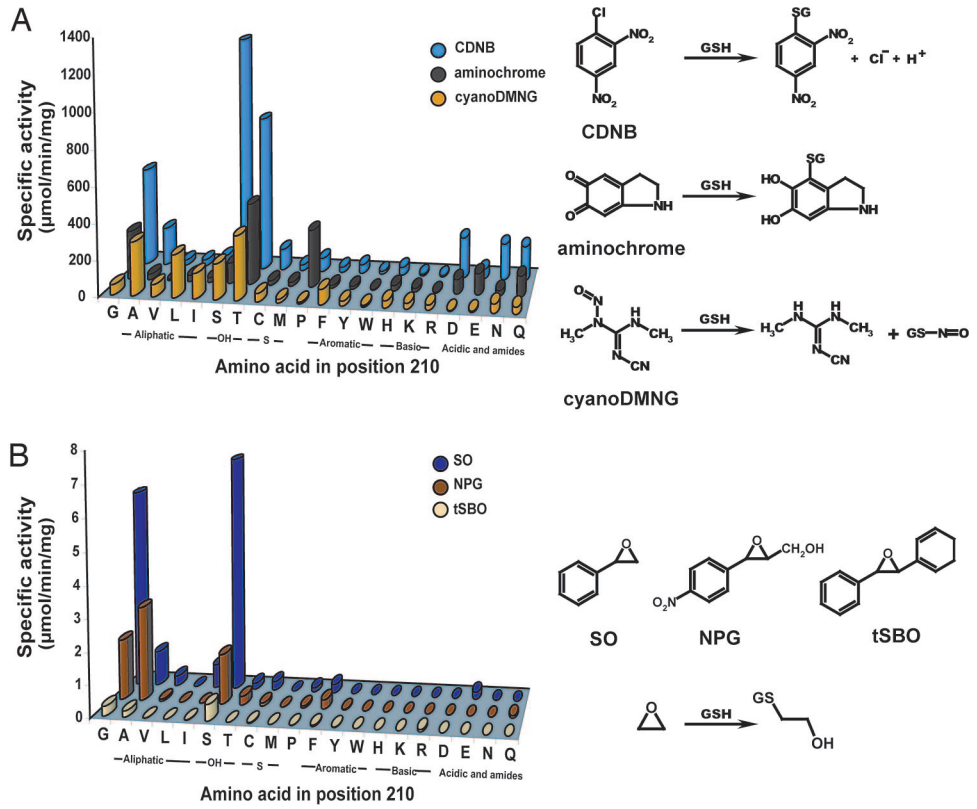

Fig. 1. Specific activities with six alternative substrates. (*A*) CDNB, cyanoDMNG, and aminochrome. (*B*) the epoxides tSBO, NPG, and SO. The different GST M2-2 variants have been divided into groups based on the character of the amino acid in position 210: aliphatic, hydroxyl-containing, sulfur-containing, aromatic, basic, and the acidic with their corresponding amides. Proline is separate because of its restricted side-chain mobility. All data are based on triplicate measurements. The standard deviations are <16% of the values, except for M2Thr and M2Val with NPG as substrate, where the standard deviations were 55% and 24%, respectively.

CDNB, cyanoDMNG, and the enantiomers of tSBO as electrophilic substrates (see Table 2, which is published as supporting information on the PNAS web site). The catalytic efficiencies (k_{cat}/K_M) with CDNB were markedly affected by replacement of residue 210, resulting from effects on both k_{cat} and K_M^{CDNB}; in contrast, the K_M^{GSH} values remained largely unchanged. In particular, the series Gly → Ala → Leu, gave rise to K_M^{CDNB} values that increased with the size of the amino acid side chain, indicating that the more bulky residues interfere with efficient substrate binding and, hence, suppress the activity with this substrate. However, a Val residue significantly lowered the rate of the chemical transition.

The cyanoDMNG activity was favored by aliphatic or hydroxyl-containing amino acid substituents at position 210, with the wild-type M2Thr displaying the highest activity (Fig. 1, and Tables 1 and 2). The steady-state kinetics revealed that the differences in catalytic activities resulted mainly from alterations in k_{cat}. The highest turnover numbers were displayed by M2Ala, M2Ser, and M2Thr, whereas a decrease in k_{cat} was observed with the more bulky aliphatic amino acids Leu and Val.

The wild-type M2Thr and most of the GST M2-2 variants had very low, if any, activity with the epoxide substrates NPG, tSBO, and SO (Fig. 1*B*, and Table 1). However, the three mutants M2Gly, M2Ala, and M2Ser had higher activities by two to three orders of magnitude with the epoxide substrates than the wild-type values, largely explained by an increase in the turnover numbers (see Table 2). Residue 210 can, hence, be considered as a molecular switch, enabling or disabling the conjugating activity with the epoxides.

Mutations of residue 210 translocate the catalytic properties of the enzyme to separate regions in the multidimensional substrate-activity space. Fig. 2 illustrates in three dimensions how the activity profile changes with respect to alternative substrates undergoing different chemical transformations. Several enzyme variants, most notably M2Ser, M2Gly, M2Pro,

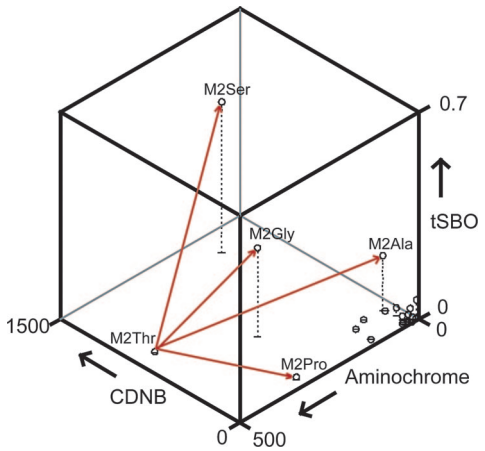

Fig. 2. A three-dimensional substrate-activity space representing the activities of the GST M2-2 variants with CDNB, aminochrome, and tSBO. Mutations of residue 210 translocate the substrate-selectivity profile of the enzyme to different regions in multidimensional substrate-activity space. Starting from the wild-type enzyme M2Thr, the profile is differentially altered in M2Ser, M2Thr, M2Ala, M2Gly, and M2Pro; some alternative mutations (not indicated) reduce the activities with all three substrates.

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M2Ala, and the wild-type enzyme M2Thr are located in different regions of the substrate-activity space. For example, the Thr-210 → Ser mutation enables activity with the epoxide tSBO without concurrent loss of the characteristic GST M2-2 activity with the orthoquinone aminochrome and with maintenance of the high activity with CDNB. The Thr-210 → Ala mutation has similar consequences as Thr-210 → Ser, with the exception that the CDNB activity is suppressed. The Thr-210 → Pro mutation disables CDNB activity without gain of activity with tSBO.

Emergence or Suppression of Alternative Chemical Mechanisms. All substitution and addition reactions studied here involve a nucleophilic attack by the thiolate of a glutathione molecule bound in the active site; however, they differ mechanistically with respect to proton transfer. A proton is released from the thiol group when glutathione binds to the enzyme. In the substitution reactions, protonation of the leaving group is not rate limiting, and, in many cases, the conjugate acid of the released group is too acidic for proton uptake. In contrast, the addition reactions are facilitated by protonation of a functional group of the substrate undergoing chemical transformation. Thus, catalysis of the addition reactions would be promoted by a proton in the activated complex of the reaction.

The substitution reactions studied can be subdivided into aromatic substitution (CDNB) and transnitrosylation (cyanoDMNG), respectively. The addition reactions segregate into Michael addition (aminochrome), and opening of a three-membered oxirane ring (epoxides). In this manner, GST activities are monitored in four dimensions of the functional "mechanism space." Our results show that point mutations in residue 210 can enable or disable different combinations of the alternative chemical mechanisms (Fig. 3). For example, the wild-type M2Thr has high activity in reactions involving substitution (CDNB and cyanoDMNG) and Michael addition (aminochrome) but low activity with epoxides. A gain of the epoxide activity with tSBO by three orders of magnitude is obtained in both variants M2Ser and M2Gly. In M2Pro, all activities except the Michael addition are disabled. These examples of variability show persuasively how diverse domains of the mechanism space could be expanded or contracted in a differential manner by simple mutations of the positively selected residue.

Comparison of M2Thr and M2Ser Structures Reveals the Key to Enabling Epoxide Activity. The most remarkable effect of the substitutions was the increase in epoxide-conjugating activity by replacement of Thr-210 with Gly, Ala, or Ser. Therefore, we solved the crystal structure of M2Ser in complex with a glutathione conjugate of styrene oxide, *S*-(*R*)-(2-phenyl-2-hydroxyethyl)glutathione, to 1.35-Å resolution. The overall structure of the M2Ser mutant is essentially the same as the wild-type structure (2GTU); the Cα-coordinates of the wild-type enzyme GST M2-2 and the M2Ser mutant have an rms distance of 0.4–0.5 Å, with all superimposed Cα-atoms within the GST monomer. Several side chains have been assigned different conformations in the two structures, presumably because of differences in crystal packing and the higher resolution in our structure, allowing us to model the side chains with higher precision. In the structure of the M2Ser mutant, the Cα-atoms of the four monomers in the asymmetric unit could be superimposed within an rms distance <0.5 Å, and the active site residues had essentially the same relative positions and conformations.

Residue 210 contributes to part of the hydrophobic substrate-binding site. Fig. 4 demonstrates that a bulky residue in position 210 would leave little space for large substrates. When the conjugate is docked into the wild-type GST M2-2 structure, the phenyl moiety collides with the methyl group of Thr-210. By the Thr → Ser replacement, this steric hindrance is relieved, which may explain the high efficiency of M2Ser with epoxide substrates. Most likely, the high epoxide activities of the M2Gly and M2Ala variants with still smaller side chains have the same explanation. Furthermore, in the structure of the wild-type GST M2-2, the methyl group of Thr-210 closes a narrow channel, partly lined with hydrophobic residues, which, in the structure of the M2Ser mutant, connects the hydrophobic binding site with the surface of the protein. It can be speculated that this channel promotes proton transfer between the active site and the surface of the protein, thus contributing to the considerably higher epoxide activity of M2Ser compared with the wild-type enzyme M2Thr. Adjacent to the portion of the binding site occupied by the conjugate of glutathione and styrene oxide, there is an additional cave-like formation that can harbor substrates of various sizes and conformations. At low contour levels, an alternative conformation of the phenyl moiety of GS-SO can be observed in the electron density map. However, the density was too weak for accurate modeling. The option of using alternative binding modes allows the active site to accommodate alternative substrates with different shapes and sizes.

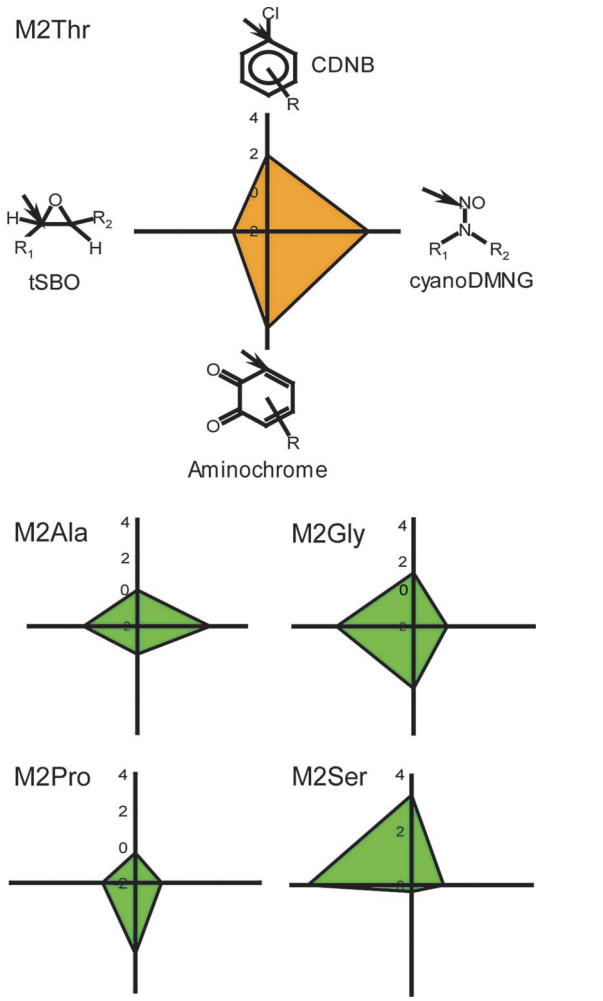

Fig. 3. Mechanism space spanned by different reaction categories: substitutions and additions. The substitution reactions can be further divided into aromatic substitution (CDNB) and transnitrosylation (cyanoDMNG). The addition reactions can be subdivided into Michael addition (aminochrome) and epoxide ring-opening (tSBO). Mutations of residue 210 disable or enable the four alternative chemical mechanisms in different combinations, depending on the amino acid replacement. The radar plots show specific activities (normalized to unit variance) with the alternative substrates.

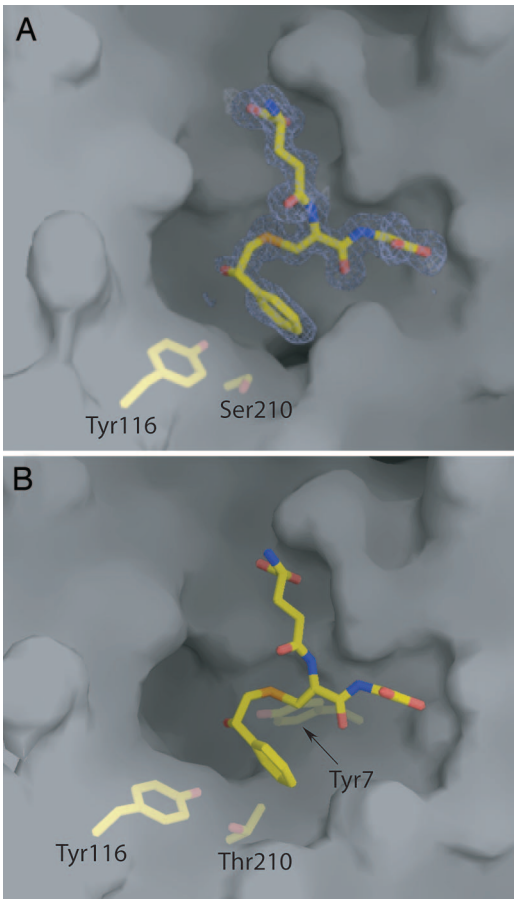

Fig. 4. Structure of the active site of GST M2-2 mutant M2Ser in complex with a glutathione conjugate of styrene-7,8-oxide. (*A*) The crystal structure of M2Ser determined at 1.35-Å resolution. Experimental 2Fo–Fc electron density map is shown for the ligand at 1σ contour level. Note the narrow channel below the phenyl ring of the ligand in M2Ser. (*B*) The modeled complex with the wild-type M2Thr structure (PDB ID code: 2GTU) showing steric interference between Thr in position 210 and the ligand.

Stability and Expressivity. To investigate to what extent substitution of residue 210 influences the thermostability of the enzyme, we determined the loss of activity of the GST M2-2 variants as a function of time (Fig. 5; and see Table 1). The thermostability differed as much as 500-fold among the enzyme variants. The most stable variants were M2Asp and M2Asn (half-lives ≈1,000 min), followed by M2His, M2Glu, M2Met, M2Gly, M2Ser, and the wild-type M2Thr, in order of decreasing stabilities. Several of the mutants showed very low thermal stability (Val, Ile, Pro, Arg, Tyr, and Trp) with half-lives <10 min; the least stable mutant was M2Val, with a half-life of 2 min. Residue 210 is located in a flexible region of the enzyme structure, and it is likely that hydrogen-bonding interactions involving residue 210 stabilize the protein. In our structure of mutant M2Ser, the hydroxyl group clearly makes a hydrogen bond to the phenolic oxygen of Tyr 116.

The effects of the mutations on the heterologous expression were determined, and the expression levels differed by an order of magnitude among the 20 GST M2-2 variants (see Table 1). The option of altering thermostability or expressivity of an enzyme can be beneficial for an organism in the course of evolution. The relative expression levels here demonstrated in bacteria may be different in human cells, but our results nevertheless demonstrate the potential of modulating the production of a protein by a point mutation.

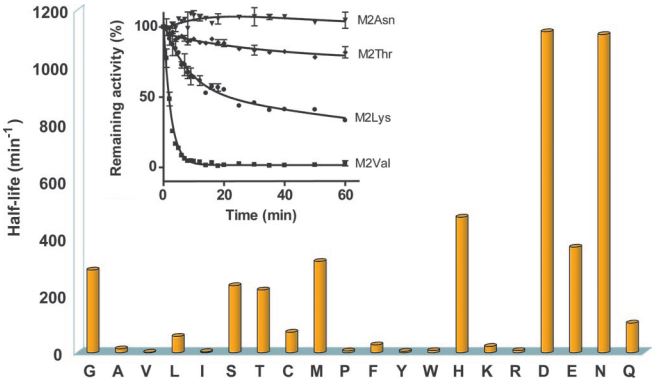

Fig. 5. Comparison of thermostabilities of the GST M2-2 variants. The orange bars represent half-lives at 48°C expressed in minutes. The insert shows four characteristic heat-inactivation curves. Half-lives differed as much as 500-fold among the mutants. M2Asn and M2Asp were the most thermostable variants, with half-lives of ≈1,000 min. The *x* axis indicates the alternative amino acids (one-letter codes) in position 210.

Naturally Evolved Variations in the Positively Selected Residue 210. Thirty-five Mu-class sequences from mammalian species were retrieved from GenBank to examine the prevalence of amino acids at the position equivalent to residue 210 in human GST M2-2 (Fig. 6). The most abundant amino acid residues at this position are Thr and Asn, followed by Leu, Ser, Ala, Gly, and Met. In our GST M2-2 scaffold, Thr, Ser, and Asn at position 210 produce stable enzyme variants with high catalytic activities, whereas the aliphatic amino acids Ala and Leu also confer high catalytic activity, albeit with lower stability.

Functional data are sparse or completely lacking for many of the known or putative Mu-class GSTs. Based on this study, Gly, Ala, or Ser at position 210 are compatible with elevated efficiency with epoxides, but other factors are obviously also instrumental in enabling catalytic activity with these substrates. Replacement of residue 210 in the Mu-class GSTs is often accompanied by other substitutions in the C terminus, in particular among the additional three positively selected residues (5) in this region (residues 205, 206, and 214). Nevertheless, it is noteworthy that, of the four human Mu-class members tested, those that contain Ser (GST M1-1) and Gly (GST M5-5) in position 210 have relatively high specific activities with tSBO (3.0 and 1.2 μmol/min/mg, respectively), whereas those containing Thr (GSTs M2-2 and M4-4) have low activities (0.0020 and 0.0016 μmol/min/mg, respectively). Possibly, the most reliable

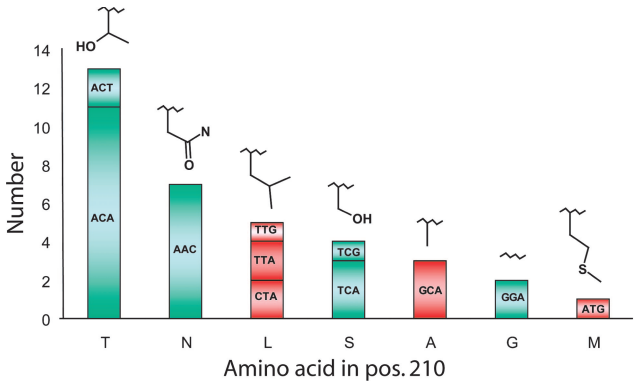

Fig. 6. Distribution of amino acids occurring naturally at position 210 among the mammalian Mu-class GSTs. Polar residues are rendered as green bars, whereas hydrophobic residues are in red bars. The frequency of the codons for the different amino acids is also indicated.

prediction of functional properties would be that mammalian Mu-class GSTs lacking any of Gly, Ala, or Ser at position 210 have low epoxide activity.

Discussion

Several positively selected codons have been identified by using different statistical models (6–8), particularly in genes involved in host–pathogen interactions, where coevolutionary processes may be responsible for rapid protein evolution (9, 10). Directed mutagenesis has confirmed the functional importance of positively selected sites in polygalacturonase inhibitor protein (11), proteorhodopsin (12), and TRIMα (13) as well as in GSTs (5). Here, we demonstrate the pivotal role of the positively selected residue 210 as a functional switch, suggesting that this residue may have played an important role in the evolution of Mu-class GST genes. The codon substitutions that give rise to favorable mutations may be produced by the change of a single base and, therefore, are close in the DNA-sequence space. For example, interchanges among Ser, Pro, Thr, and Ala can be brought about by change of the first base in one of their codons. A single point mutation of residue 210 is sufficient to alter the substrate selectivity profile of the enzyme. Hence, a duplicated gene can readily attain a novel function, which, under selection pressure, could promote fixation of the duplicated gene in the genome and be followed by optimization through Darwinian evolution.

It can be of selective advantage in evolution for detoxication enzymes to have the ability to acquire activity with novel substrates and still maintain high catalytic efficiency with substrates earlier adopted. This ability applies to the biotransformation of noxious compounds that are obligatory products of metabolism. For example, human GST M2-2 has prominent activity with orthoquinones such as aminochrome, formed from catecholamines, and their conjugation with glutathione has been proposed as a protective device against development of Parkinson's disease and other neurodegenerative conditions (14). In support of this notion, a *Drosophila* model of Parkinson's disease shows rescue of Parkin-defective dopaminergic neurons by GST expression (15). The high catalytic efficiency with orthoquinones is unique in humans to GST M2-2, and it can be argued that the acquisition of activity with novel substrates, such as epoxides, at the expense of the high activity with orthoquinones would be counterproductive. Instead, robustness of the original function in combination with the ability to adopt new substrates would be highly advantageous. In fact, this combination is what we have accomplished *in vitro*, as exemplified by variants M2Ser, M2Ala, and M2Gly. Whether the combination of high activity with both orthoquinones and epoxides actually occurs in naturally evolved GSTs is unknown, but the potential of combining prominent activities with alternative substrates is clearly demonstrated.

Some mutations suppress the catalytic activities with all of the investigated substrates but still yield proteins with high expressivity and good stability (e.g., M2Met), which may be desirable when a GST is recruited for functions other than catalysis, such as in the lens of cephalopods, in which a GST homologue acts as a crystallin (16); high catalytic activity may not be compatible with the presence of a structural protein in high intracellular concentrations. Many enzymes evolve by gene duplications and mutations of already existing enzymes, and it may, therefore, be necessary to disable the original activity in the new variant. Our results show that this can be readily accomplished in a GST. It is possible that this evolutionary potential to switch on or turn off activity with alternative substrates is particularly prominent in detoxication enzymes such as GSTs. On the other hand, the selective promotion of a particular function and concurrent restriction of others must be crucial to successful evolution of such proteins that are involved in tightly regulated networks.

Quite apart from the catalytic function, GSTs have other important tasks in the cell. Alpha-class GSTs have been identified as intracellular binding proteins carrying low-molecular-mass molecules in a manner similar to albumin in blood plasma (17). GST P1-1 was recently identified as a specific zeaxanthin-binder in the macula of the human eye (18). GSTs are also forming complexes with stress-activated protein kinases such as N-terminal c-Jun kinase and various other regulatory proteins (19, 20). Additionally, GSTs inhibit oxidative stress-induced generation of reactive oxygen species and induction of vascular endothelial growth factor A through interaction with the protease inhibitor maspin (21). It is obvious that any mutations in proteins with such multifarious functions may have wide ranging consequences in cells where they are expressed. Our finding that a point mutation in the active site of a GST can enable or disable alternative catalytic reactions without altering the properties of already established functions demonstrates a significant evolutionary plasticity useful for uncoupling of diverse activities residing in the same protein.

Materials and Methods

Construction, Expression, and Purification of GST M2-2 Variants. GST M2-2 mutants were constructed by PCRs as described in ref. 5. All primers were 24 nucleotides in length and phosphorylated at the 5′ end. Mutations were confirmed by DNA sequence analysis. The PCR products were purified, ligated, and transformed as described in ref. 5. Expression and purification of GST M2-2 variants was carried out as described in refs. 5 and 22 but eluted by using 10 mM Tris·HCl buffer containing 10 mM GSH, pH 7.8. The purified enzymes were dialyzed against 10 mM Tris·HCl buffer containing 1 mM 2-mercaptoethanol, pH 7.8, for 48 h. The yield of protein varied between 1 and 30 mg·liter^{-1} culture for the GST M2-2 variants. Protein purity was confirmed by SDS/PAGE.

Specific Activity Measurements. Six electrophilic substrates were used to investigate the effect of the mutation in position 210 of GST M2-2 (Fig. 1). Specific activities with racemic tSBO and SO were determined as described in ref. 5, with the exception that the assay buffer used with tSBO was 100 mM Tris·HCl, pH 7.2. Specific activities with CDNB (23), cyanoDMNG (23), NPG (5), and aminochrome (24) were determined by spectrophotometric assays at 30°C under standard conditions.

Steady-State Kinetic Analysis. Steady-state kinetic analysis was performed by using the electrophilic substrates CDNB, cyanoDMNG, and the enantiomers of tSBO with M2Gly, M2Ala, M2Ser, M2Thr, M2Leu, and M2Val. The conjugations of CDNB and cyanoDMNG were monitored under the same conditions as above with the concentration of GSH at a saturating level (5 mM), varying the concentration of CDNB between 0.08 and 1.5 mM, and the concentration of cyanoDMNG between 0.2 and 1.6 mM. Also, keeping the CDNB concentration at a constant level (1.5 mM), the GSH concentration was varied between 0.025 and 1 mM. The measurements with tSBO were performed as described in ref. 25. Kinetic parameters were determined by using nonlinear regression analysis. Turnover numbers (k_{cat}) are per subunit (25,600 Da).

Crystallographic Studies. The GST M2-2 mutant M2Ser was crystallized by using the hanging-drop vapor technique. In the droplet, 5 μl of protein at 10 mg/ml in 20 mM Tris·HCl, pH 8.0, was mixed with 5 μl of reservoir solution containing 100 mM Tris·HCl, pH 7.8, 2 mM DTT, 25% PEG 4000, and 1 μl of 20 mM glutathione conjugate of S-styrene 7,8-oxide. Needle-like crystals appeared in the droplets overnight. Data were collected at 100 K on the Area Detector Systems Corporation (Poway, CA) Quantum4 charge-coupled device detector at beam line ID14-1 at the European Radiation Synchrotron Facility radiation source (Grenoble, France). Crystals diffracted to 1.3-Å resolution and belonged to space group $P2_1$ with cell dimensions a = 62.1 Å, b =

67.8 Å, c = 115.7 Å, and β = 99.1°. Data were processed with the program DENZO (26) and scaled with the program SCALEPACK (26). The structure was solved by molecular replacement by using wild-type human GST M2-2 coordinates (PDB code 2GTU) as the search model in AMORE (27). The crystallographic asymmetric unit contained two GST M2-2 dimers. The model was built in the program O (28) and refined in REFMAC (29). Water molecules were added automatically with ARP/WARP (30) and manually inspected in O. Alternative conformations were modeled for several side chains. No marked differences among the four monomers of the asymmetric unit were noted in water molecules, amino acid residues, or polypeptide conformation at the active site. Scaling and refinement statistics are listed in Table 3, which is published as supporting information on the PNAS web site. Coordinates were deposited in the Protein Data Bank with PDB ID code 2C4J.

Half-Life Determination. The purified enzymes were diluted to 0.08 mg·ml^{-1} in PBS and incubated for 1 h at 48°C. Activity was measured with CDNB at 30°C under standard assay conditions. Ten-hour measurements were performed on M2Asp, M2His, M2Glu, M2Met, and M2Asn. Data were fitted to a two-phase exponential decay model by using the program PRISM 4 (GraphPad). The M2Ser and M2Phe were fitted by a one-phase exponential.

Expression Levels. Expression levels of the GST M2-2 variants were compared after parallel and simultaneous growth for 18 h in 10-ml cultures from the same batch of medium. Cells were lysed with 0.2 mg·ml^{-1} lysozyme, followed by four cycles of freeze–thawing at −80°C and 37°C. Lysates were collected after centrifugation and analyzed by using SDS/PAGE. Proteins were stained with Coomassie Brilliant blue and quantified by using IMAGEJ 1.33U (Wayne Rasband, National Institutes of Health, Bethesda).

Sequence Alignments. Protein sequences of 35 verified or putative Mu-class GSTs were retrieved from GenBank and aligned by using CLUSTALW. Fourteen of the sequences are from different primates: human (HsaGSTM1, X08020; HsaGSTM2, M63509; HsaGSTM3, J05459; HsaGSTM4, M96233; HsaGSTM5, L02321), crab-eating macaque (*Macaca fascicularis*, MfaGSTM1, AF200709; MfaGSTM2, AF200710; MfaGSTM5, AB169530), Japanese macaque (*Macaca fuscata fascata*, MfuGSTM2, AB025799; MfuGSTM3, AB025800), orangutan (*Pongo pygmaeus*, PpyGSTM2, CR859804); and chimpanzee (*Pan troglodytes*, PtrGSTM2, XP_527473; PtrGSTM3, AY369816; PtrGSTM4, XP_513625). Eighteen of the sequences have rodent origin: rat (*Rattus norvegicus*, RnoGSTM1, X04229; RnoGSTM2, J03914; RnoGSTM3, J02744; RnoGSTM4, AF106661; RnoGSTM5, U86635; RnoGSTM6, XP_215682; RnoGSTM7, BC091199), mouse (*Mus musculus*, MmuGSTM1, AF200709; MmuGSTM2, J04696; MmuGSTM3, NP_034489; MmuGSTM4, NP_081040; MmuGSTM5, U24428; MmuGSTM6, BC031818; MmuGSTM7, BC019413); guinea pig (*Cavia porcellus*, CpoGSTM1/2, AB000488); long-tailed hamster (*Cricetulus longicaudatus*, CloGSTMu, X57489); golden hamster (*Mesocricetus auratus*, MauGSTMu, M59772); and rabbit (*Oryctolagus cuniculus*, OcuGSTMu, L23766). Residual sequences are from cattle (*Bos taurus*, BtaGSTM1, AI02051), cat (*Felis silvestris catus*, FcaGSTM3, AY672094); and dog (*Canis familiaris*, CfaGSTM3, XP_537037).

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