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Shifting Substrate Specificity of Human Glutathione Transferase (from Class Pi to Class Alpha) by a Single Point Mutation

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Substrate selectivity, among glutathione transferase (GST) isoenzymes, appears to be determined by a few residues. As part of study to determine which residues are class-specific determinants, Tyr 108 (an important residue of the class Pi) has been changed to a valine, the structural equivalent of a class Alpha enzyme. Using a panel of selected substrates, "diagnostic" for either class Pi or Alpha, it is shown here that this single mutation significantly alters the catalytic properties of the class Pi enzyme and shifts the substrate specificity of the enzyme toward that of the class Alpha enzyme. © 1998 Academic Press

Glutathione transferases (GSTs, EC 2.5.1.18) are a family of enzymes involved in cellular detoxification by catalyzing the nucleophilic attack of GSH on the electrophilic centre of a number of toxic compounds and xenobiotics (1). These cytosolic enzymes have been grouped into at least seven species independent classes: (Alpha, Kappa, Mu, Pi, Sigma, Theta and Zeta) (2-7) on the basis of their amino acid sequence, substrate specificity and immunological properties (for a more updated review on the molecular properties of these enzymes see (8)). Comparison of the primary structures among the different classes reveales a very low degree of sequence identity (ranging between 20 and 30%). However, all the solved crystallographic structures of GSTs display a common three-dimensional fold and a dimeric organization (9,10). The main structural differences among these GSTs classes are in the glutathione binding site (G-site) (in particular, the helix 2 region) and in the hydrophobic substrate binding site (H-site). In spite of these differences, the G-site of each class binds GSH selectively, whilst the H-site can accommodate a large variety of substrates, different in size and apolar character. Thus, it is evident that the H-site dictates a certain degree of substrate specificity for different compounds within each class and amongst different classes. As an example, there are some substrates such as cumene hydroperoxide (CuOOH) and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-C1) which are best substrates for the Alpha-class, 1-chloro-2,4-dinitrobenzene (CDNB) is considered to be a common substrate for different classes and ethacrynic acid (EA) which is a representative substrate of the class Pi (11,12). These properties are probably due to a single or a few variations of residues which contribute to the H-site. For example, a comparison of the H-sites of classes Alpha, Mu and Pi, based on 3-D structural alignment of the crystal structures, shows that: (a) Tyr 108 (Pi enzyme) has as a counterpart Tyr 115 and Val 111, in the classes Mu and Alpha, respectively; (b) Phe 8, in class Pi, is substituted by Trp 7 in class Mu but remains invariant as Phe 10 in class Alpha; (c) Val 10 in class Pi has as a corresponding equivalent Ile 9 and Ala 12 in the classes Mu and Alpha, respectively. We have previously demonstrated that Tyr 108, located into the H-site of human GST P1-1, plays an important role via its hydroxyl group in the Michael addition of GSH to EA (13). In a search for class-specific determinants we have now mutated Tyr 108 of the human

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Abbreviations used: GSH, glutathione; GST, glutathione transferase; wt, wild type; G-site, glutathione binding site; H-site, hydrophobic substrate binding site; EA, ethacrynic acid; CDNB, 1-chloro-2,4-dinitrobenzene; NBD-C1, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; CuOOH, cumene hydroperoxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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TABLE 1

Specific Activities of Human GST P1-1, Y108V, GST A1-1 Enzymes Toward Selected Hydrophobic Substrates

Human GST	Specific activity (μmol/min/mg of protein)			
	CDNB	EA	NBD-C1	CuOOH
hGST P1-1	100 ± 10	1 ± 0.01	2.77 ± 0.1	0.03 ± 0.004
Y108V	45 ± 5	0.018 ± 0.002	8.3 ± 0.09	0.3 ± 0.03
hGSTA1-1	86 ± 5	0.1 ± 0.005	30 ± 2.5	11.2 ± 1

class Pi glutathione transferase P1-1 (GST P1-1) into valine to mimic the equivalent residue (Val 111) present in human class Alpha glutathione transferase A1-1 (GST Al-1). The mutant enzyme (Y108V) has been overexpressed in *E. coli*, purified by affinity chromatography and characterized with respect to its enzymatic properties toward different substrates.

MATERIALS AND METHODS

Expression plasmids and site-directed mutagenesis. The expression plasmid pGST-1 producing large amounts of recombinant GST P1-1 has been previously described (14). The mutant Y108V has been produced by site-directed mutagenesis according to a procedure previously described (13). The oligonucleotide 5′-AGTTGGTGACGATGAGGGA was used as mutagenic primer for the Y108V mutation. The clone of recombinant GST A1-1 has been produced and expressed as described elsewhere (15).

Protein expression and purification. GST P1-1, Y108V mutant and GST A1-1 enzymes were expressed in *E. coli* and purified by affinity chromatography as described elsewhere (16). After affinity purification all these enzymes were homogeneous as judged by SDS-PAGE (17). The protein concentration was determined by the method of Lowry (18).

Kinetic and modeling studies. The enzymatic activities were determined spectrophotometrically at 25°C with different co-substrates in a double-beam Uvicon 940 spectrophotometer (Kontron Instruments) equipped with a thermostated cuvette compartment. Enzymatic rates were corrected for the spontaneous reaction. The spectrophotometric assays with EA, CDNB, NBD-C1 as co-substrates were as previously reported (13). The activity with CuOOH as co-substrate was determined as described elsewhere (19).

Apparent kinetic parameters for different co-substrates (Table 2) were determined at fixed GSH concentration and with various co-substrate concentrations, by fitting the collected data to the Michaelis-Menton equation by nonlinear regression analysis using the Graph PAD Prism (Graph PAD Software, San Diego, CA) computer programs. Briefly, we report here the experimental conditions used for the three co-substrates assayed: (1) 0.05-0.5 mM EA in the presence of 1 mM GSH in 0.1 M phosphate buffer at pH 6.5. The apparent K_m^{GSH} was obtained at a fixed EA concentration (0.5 mM) and various GSH concentrations (from 0.05 to 1 mM). The reaction was monitored by following the increase of absorbance at 270 nm of the conjugate (EA-GS) (ε = 5700 M^{-1} cm^{-1}) (20). (2) 0.0025-0.5 mM NBD-C1 in the presence of 0.5 mM GSH in 0.1 M sodium acetate at pH 5.0; the reaction was monitored at 419 nm (ε = 14500 M^{-1} cm^{-1}) (12). The apparent K_m^{GSH} was calculated in the same conditions at a fixed NBD-C1 concentration (0.2 mM) and various GSH concentrations (from 0.002 to 0.5 mM). (3) 0.1 -2.0 mM CDNB in the presence of 5 mM GSH in 0.1 mM potassium phosphate buffer (pH 6.5) containing 0.1 mM EDTA; the reaction was monitored at 340 nm (ε = 9600 M^{-1} cm^{-1}) (20). The apparent K_m^{GSH} was also determined at a fixed CDNB concentration (1 mM) and various GSH concentrations (from 0.02 to 5 mM).

Molecular modelling was performed on a Silicon Graphics Indigo 2 workstation using the software package 'O' (21). Energy minimisation was performed using the computer package XPLOR (22). The wild-type model came from the 1.9 Å resolution crystal structure of human class Pi GST P1-1 (23). Tyr 108 was replaced with a valine residue using the "mutate" option in 'O'. The mutant model was subsequently energy minimised using 300 steps of Powell minimisation in order to relieve any residual strain caused by the change.

RESULTS AND DISCUSSION

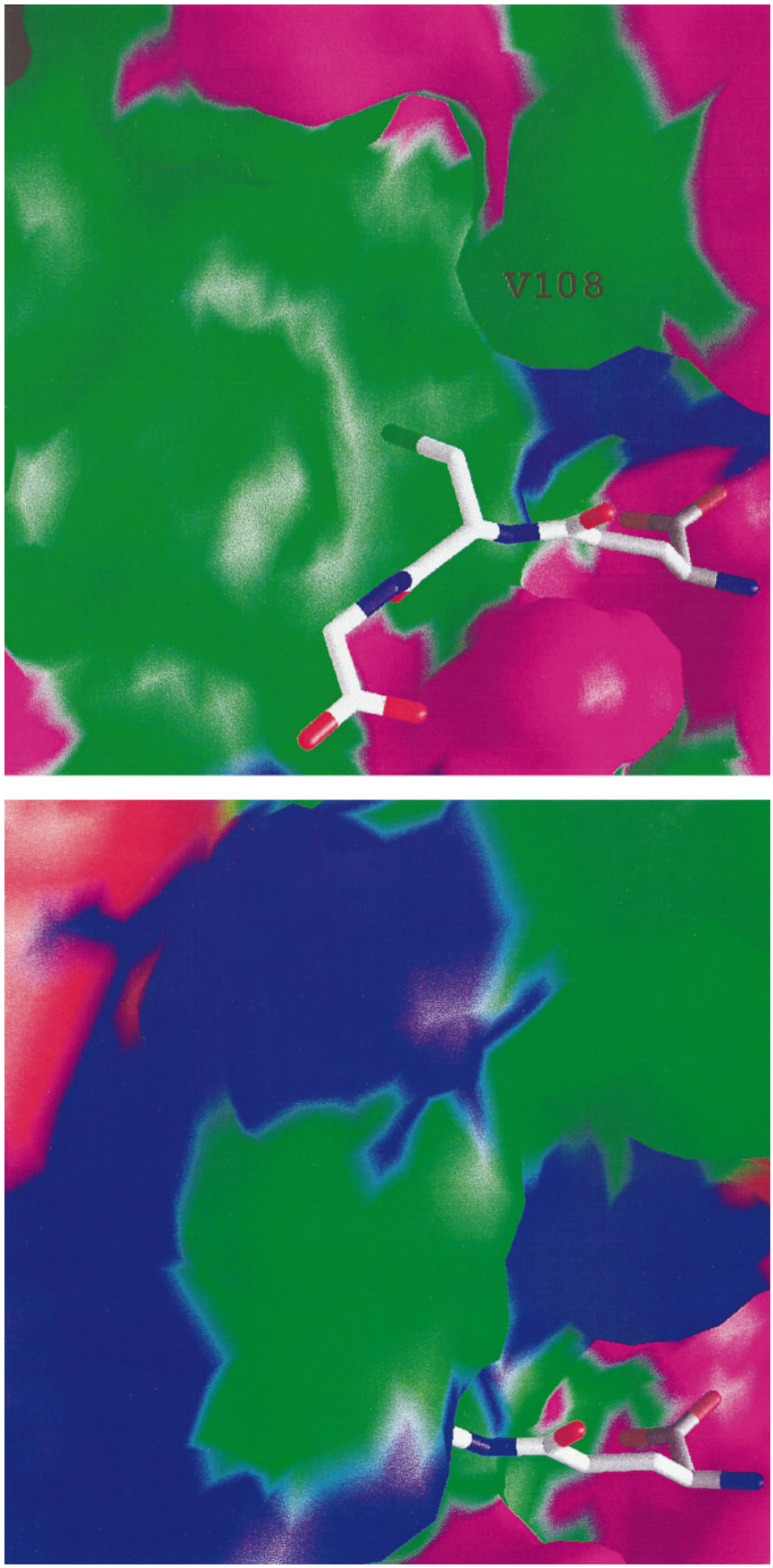
Mutation of Tyr 108 into Val increases the enzymatic activity of human class Pi GST P1-1 towards NBD-C1 and CuOOH about 3- and 10-fold respectively; whilst a decrease of about 55-fold is observed towards EA. With CDNB as general co-substrate, the specific activity of Y108V mutant is about half of that observed in the wild-type class Pi enzyme (Table 1). As reported above, all these model substrates have been used in a "diagnostic" sense to identify isoenzymes: for example, CuOOH is selective for GST A1-1 and GST A2-2 whilst

TABLE 2

Steady-State Kinetic Parameters of Human GST P1-1, Y108V, GST A1-1 Enzymes Toward Selected Hydrophobic Substrates

Substrate	Human GST	k_{cat} (s^{-1})	K_m^{GSH} (mM)	K_m^{cosub} (mM)	k_{cat}/K_m^{cosub} (s^{-1} mM^{-1})
CDNB	GST P1-1	76 ± 2	0.15 ± 0.03	1.2 ± 0.1	63 ± 2
	Y108V	60 ± 2	0.069 ± 0.02	4.9 ± 0.7	12.0 ± 2
	GSTA1-1	30.8 ± 3	0.24 ± 0.04	0.78 ± 0.14	39.5 ± 3
EA	GST P1-1	2.57 ± 0.01	0.177 ± 0.006	0.21 ± 0.02	12.2 ± 0.8
	Y108V	0.065 ± 0.02	1.07 ± 0.3	0.16 ± 0.04	0.4 ± 0.02
	GSTA1-1	0.28 ± 0.02	0.46 ± 0.12	0.13 ± 0.05	2.15 ± 0.7
NBD-C1	GST P1-1	1.1 ± 0.2	0.008 ± 0.002	0.004 ± 0.001	275 ± 12
	Y108V	6.7 ± 2	0.077 ± 0.03	0.10 ± 0.04	67 ± 8
	GSTA1-1	12.6 ± 3	0.067 ± 0.02	0.02 ± 0.005	630 ± 8

Note. Kinetic parameters reported in this Table represent the mean of at least three different experimental data sets.


V108

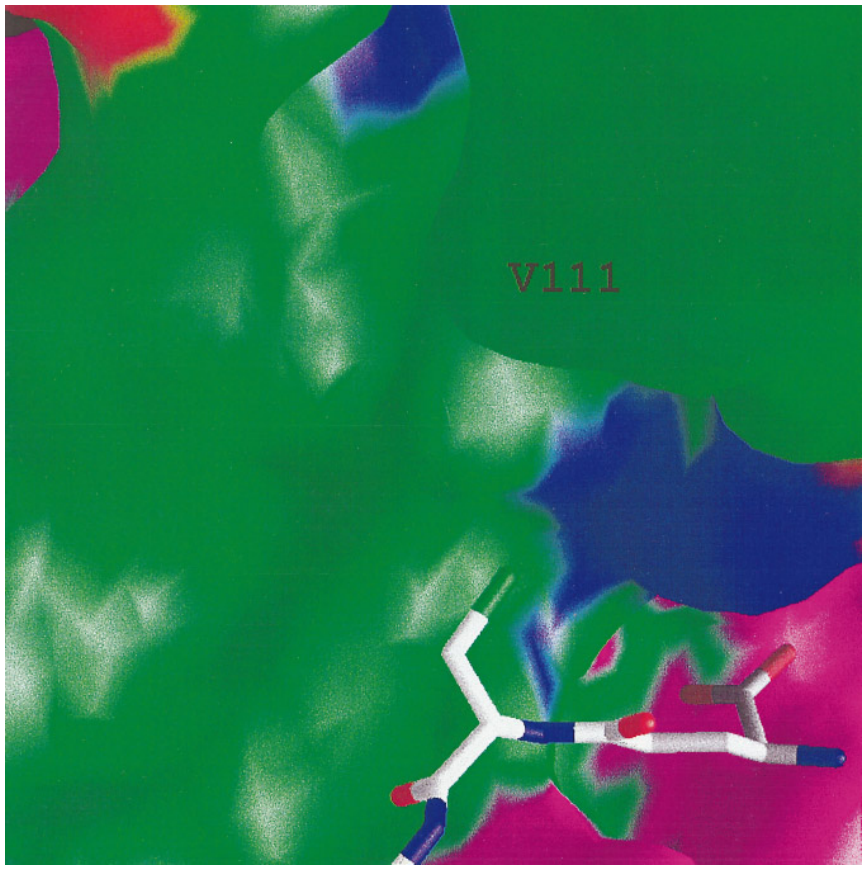


FIG. 1. Molecular surfaces of the GSTs within the vicinity of the H-site. The GSH substrate is shown in stick fashion with atoms depicted in standard colors. Green, pink, blue, and red denote hydrophobic, polar, positively charged, and negatively charged residues, respectively. The pictures were produced using GRASP (28). (a) Class Pi human GST P1-1 (23) with the Tyr 108 Val mutation indicated. (b) Class Alpha human GST A1-1 (25). (c) The same as in (b) but the C-terminal helix has been removed for clarity.

GST M1-1 and GST P1-1 isoenzymes do not possess any activity (or very low) with this co-substrate. Also NBD-C1 behaves in a similar way towards the above different GST classes, while EA is highly selective for GST-P1-1. Thus, it appears that this single mutation (Y108V) shifts the substrate specificity of the class Pi towards that of class Alpha.

The effect of this point mutation in the human GST P1-1 was further investigated by steady-state kinetic studies using the same co-substrates. The results are shown in Table 2.

With CDNB as co-substrate there is no significant change in the k_{cat} while the K_m^{CDNB} and K_m^{GSH} values were significantly altered with respect to the wt enzyme. A significant decreased affinity towards CDNB is consistent with previous observations that the aromatic ring of Tyr 108 in the GST P1-1 enzyme forms a stacking interaction with the aromatic ring of CDNB, while no specific interaction was seen for the hydroxyl portion of Tyr 108 (13, 23). On the other hand, it is worth noting that a 2-fold increase in the binding affinity towards GSH is observed in the same mutant. This indicates that the effect of a mutation in the H-site could influence also the affinity for GSH in the G-site.

With NBD-C1 as co-substrate, there is a marked increase in the k_{cat} value and a lowered affinity for both GSH and NBD-C1. Similar results were obtained in the case of the Y108F mutant (13). Plausible explanations for these observations included possible involvement of the hydroxyl group of Tyr 108 in the binding of NBD-C1, synergic modulation of both G- and H-sites caused by the mutation and/or an unfavorable modulation of k_{cat} caused by a hydrogen bond between NBD-C1 and Tyr 108. The results presented here indicate that the substitution of the aromatic ring of Tyr 108 with a hydrophobic and less bulky valine residue does not produce any additional effect on the catalytic properties observed for the Y108F mutant.

The effect of the Tyr 108 to Val mutation, using EA as substrate, is very remarkable. The enzymatic activity of Y108V mutant is decreased about 55-fold comparing to that of GST P1-1 wt and more than 5-fold as compared to that of GST A1-1. Previous studies on Y108F mutant, using the same substrate, suggested a role in catalysis for the hydroxyl group of Tyr 108 and

no effect on the binding of this substrate (13). Here we observed a k_{cat} value which is still 3-fold lower than that of Y108F mutant and a 5-fold increase of K_m^{GSH} value. Thus the main effect of this point mutation is to affect negatively the catalysis with EA, but also to significantly decrease the affinity towards GSH. These results are comparable with the catalytic properties of class Alpha GST A1-1, where the activity with EA is very low as compared to that of the class Pi enzyme (Table 1). This residual activity of GST A1-1 (Table 1) has been explained on the basis of the contribution of additional residues and/or water molecules which could play a role as the general acid in the Michael addition, in the absence of the hydroxyl group of Tyr residue (24).

The so-called "selenium independent" glutathione peroxidase activity of Y108V with an organic peroxide (CuOOH) is also noteworthy. Our results show, for the first time, that a single mutation into the H-site of the class Pi may give rise to an increased specific activity of 10-fold over the very low activity of the wt enzyme. In terms of catalytic efficiency (k_{cat}/K_m) there is a 7-fold increase of this value, determined at low substrate concentration, as compared to that of wt enzyme (0.118 mM^{-1} s^{-1} and 0.016 mM^{-1} s^{-1}, respectively).

In summary, the single point mutation of Tyr 108 to valine confirms our ideas about the strategy for converting class Pi substrate specificity to class Alpha substrate specificity although clearly more change will be required for complete conversion. The Y108V mutant shows catalytic properties which are more distant from that of class Pi GST P1-1 but approaching that of the class Alpha enzyme (Table 1). Although the activity for EA, in the Y108V mutant has been substantially lowered, consistent with the human GST A1-1 behavior, the activity with NBD-C1 and CuOOH as substrates, should be increased further 4- and about 30-fold, respectively, in order to obtain comparable catalytic properties of the class Alpha enzyme. Thus it is expected that other residues and/or protein segments in the H-site are involved in dictating this class specificity towards the above substrates. An inspection of the H-sites of both GST P1-1 and GST A1-1 isoenzymes, using the available crystal structures (23, 25) reveals striking differences which could help explain the results presented here (Figure 1). The H- site of GST P1-1 is located in a surface canyon with Ile 104, Gly 205, Tyr 108 and Asn 204 forming one wall, Phe 8 and Val 35 forming the other wall and Tyr 7, Pro 9, Val 10 and Gly 12 forming the base. Although most of these residues are hydrophobic, the canyon is completely accessible to solvent and the site can be subdivided in two distinct regions: one, which is more hydrophobic and consists of Tyr 7, Phe 8, Pro 9, Val 10 and Gly 12 and one, which is more hydrophilic, contributed by Ile 104, Tyr 108, Asn 204 and Gly 205 residues (23). The latter region is filled with a network of water molecules suggesting this region interacts with polar substrates or polar moieties of hydrophobic substrates when bound to the H-site. In contrast, the H-site of class Alpha GST A1-1 is very hydrophobic and smaller in size as compared to that of class Pi. An additional helix (helix 9) coming from the C-terminus seems to be very flexible and acts to form a lid over the H-site, in the presence of substrate (22, 23). Thus, additional residues with changes in hydrophobicity and size are expected to play a role in varying substrate specificity from class Pi to class Alpha. Mannervik and coworkers have attempted to redesign the active site of GST A1-1 by the phage display technique (26). A quadruple mutant has been produced which showed altered catalytic properties with respect to the same substrates used in this work. Thus, different approaches may be used in attempting to convert substrate specificity of an enzyme. Our results suggest that, in an ideal reconstruction of the H-site of the GST A1-1, using GST P1-1 as template, one should consider the importance of this minimal mutation (Tyr108 to Val) as a prerequisite to build up this newly engineered enzyme. From the evolutionary point of view, the likelihood of reconstructing, among the different classes, a common phenotype is greater than the likelihood of accurately reconstructing an ancestral sequence (27).

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