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Tyrosine 50 at the Subunit Interface of Dimeric Human Glutathione Transferase P1-1 Is a Structural Key Residue for Modulating Protein Stability and Catalytic Function

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The dimer interface in human GSTP1-1 has been altered by site-directed mutagenesis of Tyr50. It is shown that the effects of some mutations of this single amino acid residue are as detrimental for enzyme function as mutations of Tyr8 in the active site. The dimeric structure is a common feature of the soluble glutathione transferases and the structural lock-and-key motif contributing to the subunit–subunit interface is well conserved in classes alpha, mu, and pi. The key residue Tyr50 in GSTP1-1 was replaced with 5 different amino acids with divergent properties and the mutant proteins expressed and characterized. Mutant Y50F is an improved variant, with higher thermal stability and higher catalytic efficiency than the wild-type enzyme. The other mutants studied are also dimeric proteins, but have lower stabilities and catalytic activities that are reduced by a factor of 10^2–10^4 from the wild-type value. Mutants Y50L and Y50T are characterized by a markedly increased K_M value for GSH, while the effect is mainly due to decreased k_{cat} values for mutants Y50A and Y50R. In conclusion, residue 50 in the interface governs both structural stability and catalytic function.

Key Words: **glutathione transferases; structural motifs; mutagenesis; subunit interactions; catalysis.**

The soluble glutathione transferases (GSTs) are dimeric proteins composed of identical or structurally related subunits (1, 2). Each subunit of 25 kDa is built of two domains and contains a complete active site consisting of a G-site (glutathione binding site) and an H-site (hydrophobic substrate binding site) (3). Several studies of rat and human GSTs have shown that the individual subunits are catalytically independent (4–6). Thus, the properties of heterodimeric GSTs can be predicted from those of the corresponding homodimers. In contrast to these results, it has been reported that the human enzymes GSTP1-1 (7, 8) and GSTA4-4 (9) under some special conditions display cooperativity between the subunits. These findings raise questions about the nature and functional significance of the oligomeric structure of GSTs.

More than 100 GST sequences are currently known, thus representing a superfamily of closely related enzymes (10). GSTs can be found in all oxygen-utilizing species and their role seem to be the detoxication of a broad range of electrophilic compounds (11–13). The GSTs are divided into classes based on primary structure similarities and earlier on substrate specificities (14). In spite of limited similarities of sequences from different classes, GSTs adopt the same fold as judged by the available 3D structures (15, 16). Few amino acid residues are strictly conserved among the GSTs. Conserved residues forming local structural motifs of importance to the folding of GSTs have been identified (17–19). The contributions to catalysis of several amino acid residues, shown by 3D analysis to be parts of the active site, have been clarified (for a review, see 13). Amino acid residues in the dimer interface are not universally conserved in GSTs, rather there is a class-specific conservation. However, a particular lock-and-key structure is a common feature of GSTs of the alpha, mu and pi classes (20–22). The sigma and theta classes, from which classes alpha, mu, and pi have evolved, have another interface topography with fewer protruding residues and a more hydrophilic character (23, 24). Hydrophobic interactions dominate in the lock-and-key motif. The key residue, always an aromatic amino acid, located in domain I in one subunit is wedged into a lock formed by helices α4 and α5 in domain II in the other subunit (Table 1). The significance of the individual amino acid residues forming the lock and key motif is not known in any molecular detail.

In the present investigation we have examined the role of the key residue Tyr50 in human GSTP1-1, a member of the pi class. Five mutants were expressed,

Abbreviations used: hGSTP1-1, human class pi glutathione transferase; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene.

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TABLE 1

The Lock-and-Key Residues in Class pi GSTs from Different Species

	Key-residue	Lock-residues				
Enzyme[1]	Position[2] 50	92	96	129	130	133
Class pi						
human, hGSTP1-1	Y	M	G	P	F	L
mouse, mGSTP1-1	Y	M	G	P	F	L
bovine, bGSTP1-1	Y	M	G	P	F	L
hamster	Y	M	G	P	F	L
goat	Y	M	G	P	F	L
toad, bbGSTP1-1	Y	M	G	H	F	L
toad, bbGSTP2-2	F	M	G	Y	F	L
nematode	F	M	G	K	F	L
porcine, pGSTP1-1	F	M	G	P	F	L
Class alpha						
human, hGSTA1-1	F	M	G	A	F	V
Class mu						
human, hGSTM1-1	F	I	Q	L	Y	F

Note. Corresponding residues in GSTs representing the alpha and mu classes are shown for comparison.

[1] Data are compiled from references (25–27) and information available on the Internet.

[2] Numbering of the residues is based on the human GSTP1-1 sequence where the initiator Met is regarded as 1. The corresponding amino acid residues in other GSTs have other sequence positions.

purified and characterized. It is shown that some mutations in this position are as detrimental to the function of the enzyme as mutations of residues directly involved in catalysis.

MATERIALS AND METHODS

Construction of mutants. Single-point mutants of human GSTP1-1 (28) were generated site-specifically using inverted PCR. The following primers (GIBCO BRL Life Technologies) were used for the mutations: Tyr50Ala/Thr, 5′-TCCTGCCTA***RCY***GGGCAGCTC, where R = A + G and Y = C + T. Tyr50Leu, 5′-TCCTGCCTA***CT-G***GGGCAGCTC; Tyr50Phe, 5′-TCCTGCCTA***TTC***GGGCAGCTC; Tyr50Thr, 5′-TCCTGCCTA***ACC***GGGCAGCTC; the reverse primer had the sequence: 5′-GGCTTTGAGTGAGCCCTCCTGC. Altered codons are shown in bold italics. The PCR reaction mixture contained 0.8 μM of each phosphorylated primer, 0.2 mM dNTPs, 2.5 units of *Pfu* DNA polymerase (Stratagene), the buffer supplied with the enzyme and various amounts of template, the expression clone pKHP1 (28). The temperature program started with 94°C for 10 min and was followed by 25 cycles of 94°C for 1 min, 55–65°C for 1 min and 72°C for 9 min. The program terminated with a reaction at 72°C for 30 min. The PCR product was recovered from agarose gel after electrophoresis, ligated and used to transform competent *E. coli* XL-1 Blue cells. The complete cDNA encoding GSTP1-1 was sequenced to verify that no undesired mutations had been introduced through the PCR reaction. A histidine tag of six residues was added to the N-terminus of the proteins by PCR following the same procedure as above.

Expression and purification. Wild-type GSTP1-1 and mutants were expressed in *E. coli* at 37°C as previously described (28). All enzyme variants could be purified in a single chromatographic step using Ni-IMAC (Amersham Pharmacia Biotech) and eluted with an imidazole gradient. The eluted proteins were dialyzed towards a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and 2 mM DTT. The same buffer was used for gel filtration. Purity of the enzymes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Kinetic studies. Specific activity and kinetic parameters for the mutants as well as for the wild-type GSTP1-1 were determined by use of the conjugation reaction between glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) catalyzed by the enzymes (29). The standard assay conditions for specific activity measurements are 1 mM GSH, 1 mM CDNB in 0.1 M NaPi buffer, pH 6.5. For determination of V_{max}^{GSH} and K_M^{GSH}, CDNB was kept constant at 1 mM while GSH concentrations were 0.02-120 mM. For determination of k_{cat}/K_M^{CDNB}, GSH was 5 mM and CDNB 0.025-0.1 mM. The reaction was monitored at 340 nm and the data obtained were analyzed using GraphPad Prism (GraphPad Software Inc.).

Thermal stability. Thermal stability was measured as a function of time. The different enzyme variants were incubated (1 μM) at 50°C and aliquots were assayed for activity in the standard system at different time-points. The experiment was repeated with 5 mM GSH present in the incubation mixture. Half-lives for the enzyme variants represent the time of incubation when there is 50% residual activity.

RESULTS

Expression and purification. The Tyr50 mutants could all be expressed in *E. coli* at 37°C, but the yields of soluble protein varied significantly for the different enzymes. The wild-type enzyme and mutant Y50F represented approximately 20% of total soluble protein in the bacterial lysate. For the Y50L and Y50T mutants this value was 15%, while the Y50A and Y50R mutants were expressed at a lower level (about 5%).

Usually GSTP1-1 is conveniently purified in a single chromatographic step using affinity chromatography on immobilized GSH. It was found that the affinity for this matrix was strongly reduced for the Tyr50 mutants, with the exception of mutant Y50F. A histidine-tag was therefore added to the N-terminus of the proteins. The histidine-tag was added also to the wild-type enzyme and the Y50F mutant in order to avoid ambiguities in the comparison of the variant proteins. The kinetic properties of the histidine-tagged wild-type GSTP1-1 did not differ significantly from unmodified wild-type enzyme purified by other means.

Structure and stability. The dimeric state of all GSTP1-1 variants was established by gel filtration (results not shown).

The nature of the side-chain in position 50 is of importance for thermal stability as shown in Table 2. Removal of the phenolic hydroxyl group of Tyr50, which gives mutant Y50F, leads to a significantly more stable enzyme with a 2.5-fold increase in half-life. On the other hand, replacement of the aromatic group with an aliphatic side-chain reduces the thermal stability by the same factor (mutants Y50L and Y50T). A very small or a strongly hydrophilic amino acid side-chain is hardly tolerated at all. Mutants Y50A or Y50R were highly unstable and no activity whatsoever could be detected after incubation at 50°C. Addition of GSH has a stabilizing effect, most notable for the wild-type

TABLE 2

Thermal Stability of GSTP1-1 and Mutants

Enzyme	Half-life (min)	Half-life after incubation in the presence of 5 mM GSH (min)
Wild-type	28	92[1]
Y50F	70	>150
Y50L	10	13
Y50T	12	14

Note. Half-lives represent the time of incubation after which 50% activity remains measured in the standard assay system, the conjugation between CDNB (1 mM) and GSH (1 mM; 20 mM for mutants Y50L and Y50T).

[1] Data from (30).

and Y50F variants and to a lesser extent for mutants Y50L and Y50T.

Catalytic properties. The catalytic properties of GSTP1-1 and mutants are summarized in Table 3. It is shown that the residue in position 50 has a strong influence on specific activity and the other kinetic parameters investigated here. The effect is most conspicuous for the Y50A and Y50R mutants. These mutants display less than 0.1% of the wild-type specific activity and k_{cat}/K_M^{GSH} values are decreased even more. These effects result from a combination of reduced k_{cat} and increased K_M^{GSH} values. For the Y50L and Y50T mutants on the other hand, a markedly raised K_M^{GSH} is the primary cause for the lowered catalytic efficiencies, while the V_{max} values are about half of the wild-type value. The kinetic parameters for mutant Y50A should be looked at with some caution, because of the difficulties in characterizing this unstable enzyme. In contrast to the other mutations, it is clear that removal of the Tyr hydroxyl group is favorable for enzyme activity. Mutant Y50F has significantly higher specific activity and V_{max}^{GSH}, but only a slightly raised K_M for GSH.

DISCUSSION

A number of studies on the effects of site-directed mutagenesis of GSTs have been carried out. Most of these studies have focused on amino acid residues that are involved in substrate binding and catalysis. The first and most important residue targeted was the active-site Tyr, which stabilizes the thiolate form of GSH (31–33). In human GSTP1-1 this Tyr8Phe mutation reduces the specific activity to 0.3% of the wild-type value (32). Recently, also residues of importance to structure and folding have been identified in GSTs (17–19). The present investigation shows that mutation of a single amino acid residue serving a structural function at the dimer interface can have an impact on the catalytic function of an enzyme, which is as profound as altering an active-site residue.

Subunit-subunit interactions in classes alpha, mu and pi GSTs are partly maintained by a lock-and-key motif (Table 1). The key residue, which is Tyr in most class pi structures and Phe otherwise, is wedged in a lock formed by five amino acid residues located in domain II in the other subunit (Table 1). It has been suggested that the dimeric structure serves to stabilize the individual subunits and that a stable monomer could not exist (34). However, the results from different equilibrium unfolding studies are somewhat contradicting. The presence of a structured monomer during unfolding (35) and a contrasting two-state mechanism (34, 36) excluding such an intermediate have been suggested.

Tyr50 in GSTP1-1, and the corresponding residue in other isoenzymes, is located in a loop between the α2-helix and the β3-strand in a flexible region of the protein. Parts of this region contribute to one side of the G-site. The first 3D structures of GSTs were of complexes of the enzyme and various substrates or inhibitors. Later, it was shown that the crystal structures of the apoenzymes were essentially identical except for GSTP1-1 (37). In contrast to other GSTs, binding of GSH influences the conformational flexibility of GSTP1-1. The flexible region is locked in place and Tyr50 becomes involved in 15 different interactions with the opposing subunit, while the same residue in the apoenzyme is involved only in 8 such interactions. Upon binding of GSH, the hydroxyl group of Tyr50

TABLE 3

Specific Activities and Michaelis-Mentens Parameters for GSTP1-1 and Mutants

Enzyme	Specific activity (μmol · min^{-1} · mg^{-1})	k_{cat}/K_M^{CDNB} (mM^{-1} · s^{-1})	k_{cat}/K_M^{GSH} (mM^{-1} · s^{-1})	K_M^{GSH} (mM)	V_{max}^{GSH} (μmol · min^{-1} · mg^{-1})
Wild-type	110 ± 6	118 ± 4	169 ± 12	0.42 ± 0.10	224 ± 11
Y50F	140 ± 5	384 ± 19	320 ± 14	0.53 ± 0.16	239 ± 19
Y50A	0.015 ± 0.005	0.028 ± 0.014	0.005 ± 0.001	2.1 ± 0.7	0.075 ± 0.01
Y50L	1.2 ± 0.1	4.2 ± 0.2	0.54 ± 0.04	70.4 ± 10	119 ± 10
Y50R	0.1 ± 0.03	0.16 ± 0.04	0.02 ± 0.004	1.8 ± 0.4	0.22 ± 0.02
Y50T	1.0 ± 0.1	4.0 ± 0.2	0.36 ± 0.04	67.8 ± 9.5	130 ± 8

Note. All measurements were performed at 30°C as described under Materials and Methods. The k_{cat}/K_M values were calculated from the initial linear part of the saturation curve at low concentrations of the varied substrate.

comes closer to the carbonyl group of Met92 in the other subunit and a hydrogen bond is formed (37). It has also been shown that binding of GSH can protect GSTP1-1 from proteolysis (38). The unliganded enzyme is a substrate for chymotrypsin and the cleavage site is between residues Tyr50 and Gly51 (39). The accessibility of this region to proteases indicates that the two subunits are not locked tightly together and that Tyr50 cannot be completely buried. Further evidence for conformational changes in GSTP1-1 comes from the oxidation of cysteine residues. Cys48 is readily forming a disulfide bond with Cys102 under special conditions, leading to an inactive enzyme (40, 41). A requirement for this modification to take place is that the enzyme is in a flexible unliganded state. The induction of conformational changes in GSTP1-1 by substrate binding has been suggested as a way of regulating activity of this particular GST (42). The multifunctional role proposed for the residues in the dimer interface prompted the present investigation to elucidate the significance of the key residue Tyr50.

Apparently, there is not an absolute requirement for Tyr or Phe in the key position for dimerization to take place, as judged from the results of gel filtration (data not shown). Also the most severely affected mutants Y50A and Y50R form dimers readily. An amino acid residue with an aromatic side-chain is favored, since replacement with a bulky hydrophobic residue such as Leu is detrimental to both stability and catalysis. This preference for aromatic residues in protein-protein interactions has been statistically verified (43) and is not unique to the GSTs. According to Argos (44), aromatic residues are particularly well suited to serve as "glue" in such interactions.

The presence of Tyr in the key position provides for additional electrostatic interactions in the dimer interface compared to the more frequently occurring Phe. With this in mind it may not be surprising that removal of the hydroxyl group as in mutant Y50F leads to a less conformationally restricted enzyme which also has a higher catalytic activity. K_M for GSH is hardly affected at all; the effect is almost solely due to an increase in k_{cat}. The reason for the significant increase in thermal stability is less obvious. Mutant Y50F is more resistant than the wild-type enzyme to denaturation by urea and guanidinium chloride as well (data not shown).

Mutants Y50L and Y50T are very similar in their properties although Leu and Thr have distinct functionalities. Both mutants are characterized by a markedly increased K_M for GSH (160–170 fold), while k_{cat} is only reduced to approximately half of the wild-type values. The thermal stability is down about 2.5 times, but at 4°C these mutants are very stable over time.

During the preparation of this manuscript a similar study was published by Dirr and co-workers (45). Here, GSTA1-1, a human alpha class enzyme, was studied and Phe51, the key residue, was replaced by Ser. The effects are similar to those obtained for the Y50T and Y50L mutants of GSTP1-1 described in the present investigation in that the effect of the GSTA1-1 mutation was almost entirely due to the increase in K_M for GSH (70 fold). The effects on stability and k_{cat} were very modest.

Introduction of a small (Y50A) or very hydrophilic (Y50R) amino acid residue in place of Tyr50 leads to almost complete inactivation of GSTP1-1. For these mutants the effect is primarily due to decreased k_{cat} values, while K_M for GSH is less affected. Although these latter mutants are very low in activity, the rate equation used for analysis and determination of kinetic parameters fitted very well to the experimental data. This was also the case for the wild-type and mutant Y50F enzymes, demonstrating the expected hyperbolic substrate dependence. For the mutants Y50L and Y50T, on the other hand, there was a significant deviation from a hyperbola and a sigmoidal curve-shape was evident. The explanation for the sigmoidicity is presumably that binding of GSH to one subunit stabilizes the structure such that the affinity for GSH increases in the neighboring subunit.

The lock residues are strictly conserved among the mammalian class pi GSTs and the key position is held by an aromatic residue. These conserved motifs explain at least partly why heterodimer formation can occur only with subunits from the same class. The results of the present study show that amino acid residues with very different properties can function as key residues for subunit dimerization, but for efficient enzymes the requirements are more specific. In GSTP1-1, as well as in many other isoenzymes, a single active-site residue, Asp99, in one subunit interacts with the bound GSH in the G-site of the other subunit (21), underlining the importance of the dimeric structure of GSTs. The present investigation shows that a single amino acid residue that is not directly part of the active site can have a great impact on both enzyme function and protein stability. This finding provides evidence for a regulatory role played by the interface-forming amino acid residues.

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