The High Activity of Rat Glutathione Transferase 8–8 with Alkene Substrates Is Dependent on a Glycine Residue in the Active Site

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Rat glutathione transferase (GST) 8–8 displays high catalytic activity with α,β-unsaturated carbonyl compounds, including lipid peroxidation products such as 4-hydroxyalkenals. The catalytic efficiency of the related class Alpha GST 1–1 is substantially lower with the same substrates. Chimeric enzymes were prepared by replacing N-terminal subunit 8 segments of different lengths (6, 25, or 100 residues) with corresponding sequences from subunit 1 using recombinant DNA techniques. The chimeric subunit r1(25)r8, containing 25 amino acid residues from subunit 1, had the same low activity with alkenal substrates as that displayed by subunit 1. Mutation of Ala-12 into Gly in r1(25)r8 gave rise to the high alkental activity characteristic of subunit 8, showing the importance of amino acid residue 12 for the activity. However, other structural determinants are also essential, as demonstrated by the corresponding Ala-12 → Gly mutation in subunit 1, which did not afford high alkental activity. The results show that a single point mutation in a GST subunit may give rise to a 100-fold increase in catalytic efficiency with certain substrates. Introduction of such mutations may have contributed to the biological evolution of GST isoenzymes with altered substrate specificities and may also find use in the engineering of GSTs for novel functions.

The cytosolic glutathione transferases (GSTs)1 are homologous proteins that have been divided into four distinct structural classes (Mannervik et al., 1985; Meyer et al., 1991). Members of a given class usually have amino acid sequence identities >70%, whereas isoenzymes of different classes have sequence identities <35% (Mannervik and Danielson, 1988). Crystal structures of members of classes Alpha (Sinning et al., 1993), Mu (Ji et al., 1992; Raghunathan et al., 1994), and Pi (Reinem et al., 1991, 1992; Dírr et al., 1994; García-Sáez et al., 1994) have been determined, and the similarities in protein fold among members from the different classes as well as the differences in the detailed topography of the secondary structures are fully consistent with the view that the GST classes are branches on a common evolutionary tree (Mannervik, 1985).

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1 The abbreviations used are: GST, glutathione transferase; PCR, polymerase chain reaction; CDNB, 1-chloro-2,4-dinitrobenzene.

Attempts have been made to classify glutathione transferases on the basis of activities with different substrates (Boyland and Chasseaud, 1969), but in general substrate specificities are largely overlapping, and members within the same structural class can diverge very significantly in their substrate preferences. For example, the human class Alpha enzymes GST A1–1 and GST A2–2 differ only in 11 out of the 222 amino acids encoded per subunit (Lai et al., 1984). Only four of the variant amino acids are located at the active site (Sinning et al., 1993). These limited structural differences appear to govern the significant differences in substrate specificities reported (Chow et al., 1988; Burgess et al., 1989).

In the rat, a class Alpha isoenzyme, GST 8–8, has been identified, which is characterized by particularly high catalytic activity with 4-hydroxyalkenals (Jönsson et al., 1986) and other activated alkenes (Stenberg et al., 1992). The substrates include many highly cytotoxic α,β-unsaturated carbonyl compounds produced by lipid peroxidation, radical reactions, and other processes elicited by oxidative stress (Berhane et al., 1994). In terms of protein design as well as from the evolutionary viewpoint, it is of interest to elucidate the structural basis for the high catalytic activity of GST 8–8 with these pathophysiologically important substrates. We have previously demonstrated that fully functional chimeric GSTs can be constructed by replacing the C-terminal one-third of a human GST structure with the corresponding segment of a rat GST (Mannervik et al., 1990; Björnstedt et al., 1992). In the present investigation it was therefore decided to construct chimeric GSTs from segments of rat GST 8–8 and rat GST 1–1 in an attempt to locate amino acid residues essential for the high catalytic activity with alkene substrates. GST 1–1 is a class Alpha enzyme that is 59% sequence identical with GST 8–8 (Stenberg et al., 1992). Its catalytic activity with long chain 4-hydroxyalkenals is 1 order of magnitude lower than that of GST 8–8, but the catalytic efficiency of GST 1–1 is still 1 order of magnitude higher than the other rat GSTs investigated (Danielson et al., 1987). The results of the present investigation show that the characteristic high catalytic efficiency of GST 8–8 with activated alkenes is critically dependent on Gly-12 in the active-site region.

MATERIALS AND METHODS

Construction of an Expression Plasmid for GST r1(100)r8—Expression vectors for the chimeric GSTs were prepared by taking advantage of unique restriction sites, either naturally present in the cDNA or introduced by specific primers, and the polymerase chain reaction (PCR). The expression plasmid for rat class Alpha subunit 1, pKRA1 (Björnstedt et al., 1992) was digested with BglII and SalI to remove 366 base pairs of the 3′-coding region of the cDNA. The corresponding DNA segment in pKGRAB (Stenberg et al., 1992) coding for amino acids 101–222 of rat class Alpha subunit 8, was amplified using PCR. The primers used (synthesized at Operon Technologies Inc. Alameda, CA) had ends compatible with the BglII-Sall-digested pKRA1 vector (forward primer, 5′-CCA AGA TCT GAT GAT GAT TATC-3′; reverse primer, 5′-AAGTCGAC(TT)2-3′). The conditions for PCR were as follows: 0.2 mM dNTPs, 1.5 mM MgCl2, 1 μM of each primer, and 2.5

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units of Taq polymerase. Temperature cycle was as follows: 1 min at 95 °C, 2 min at 54 °C, and 3 min at 72 °C repeated 30 times. After cloning of the amplified fragment into pKR1A to give pKR1(100)R8, the construct was verified by dye sequencing (Sanger et al., 1977).

Construction of an Expression Plasmid for GST r1(25)r8—A restriction site (PstI) already present in the rat subunit 1 cDNA was introduced by being cloned in the coding position in the rat subunit 8 cDNA by PCR. This restriction site was then used as the fusion point for the two cDNA segments to give pKR1(25)R8 (forward primer, 5'-GGC TGC AGC TGG AGT GGA GTT; reverse primer, 5'-AAGTGCACT(T)23-3').

Construction of an Expression Plasmid for GST r1(6)r8—The expression vector for GST r1(6)r8 was assembled by replacing the first 12 codons in pKGTRA8 with synthetic oligonucleotides containing the six 5'-terminal codons of the subunit 1 cDNA. A DNA fragment encoding amino acids 13–222 of rat subunit 8 was generated by PCR using pKGTRA8 as template. The reverse oligo(dT) primer, described above, was used together with a forward primer, containing EcoRI and EdCl restriction sites, for the amplification (forward, 5'-TGG AAT TCG CCG CCG TAT GGA GTC GTC GAT-3'). The resulting DNA fragment was cloned into the EcoRI-Sacl sites of M13mp18 (Boehringer). Two partially overlapping linkers (5'-TTG AAT TCA TCT TCT GGA AGA CAG TGC TT-3') and (5'-AAC GGC GGC GGC CCT GCT AGT AAA GCA GCA GTG-3') were annealed and extended by Sequenase (Amersham Corp.). The resulting double-stranded DNA fragment, encoding the missing N-terminal 12 amino acids, was cloned between EcoRI and EdCl of the M13 construct containing the truncated 8 cDNA, resulting in M13mp16r(6). The complete unit, coding for r1(6)r8, was then transferred from M13mp16r(6) and cloned down-stream of the inducible tac promoter in the expression plasmid pKK223-3 (Pharmacia Biotech Inc.) to give the expression vector pKR1(6)r8.

Construction of GST r1(25)r8 HR and N11Q—Single-stranded DNA of a M13mp19 clone with the expression unit (tac promoter and coding sequence) of pKR1(25)r8 was used as a template for site-directed mutagenesis by the method of Taylor et al. (1985) with specific oligonucleotides (H8Y, 5'-ATT GAA GTA GGA AGA CAG TGC TT-3'; N11Q, 5'-CTG GCC CGG GC-3'), which were annealed and extended by Sequenase (Amersham Corp.); this successful approach led to the isolation of a clone capable of distinguishing rat sequences from human sequences in chimeric constructs (cf. Björnstedt et al., 1992). The amino acid substitutions afforded by the construction of the chimeras appear by comparing the parental subunits (Fig. 1). The chimera r1(25)R8 was also subjected to site-directed mutagenesis, to make it similar to GST 8–8, in order to assess the contribution of single amino acids (H8Y, N11Q, and A12G) to catalysis and to binding of the electrophilic substrate.

RESULTS

Expression and Purification of Chimeric and Mutant GSTs—A series of chimeras was constructed from the parental GST 1–1 and GST 8–8 to probe segments of the primary structure as determinants for specificity and catalytic efficiency toward α,β-unsaturated carbonyl compounds. Three such chimeras, r1(6)r8, r125)r8, and r1100)r8, were obtained in which 3, 11, and 45%, respectively, of the sequence beginning from the N terminus is derived from subunit 1 and the remainder up to the C terminus is derived from subunit 8; the number within parentheses denotes the amino acid residue at the fusion point between the two segments; “y” is used to distinguish rat sequences from human sequences in chimeric constructs (cf. Björnstedt et al., 1992). The amino acid substitutions afforded by the construction of the chimeras appear by comparing the parental subunits (Fig. 1). The chimera r1(25)R8 was also subjected to site-directed mutagenesis, to make it similar to GST 8–8, in order to assess the contribution of single amino acids (H8Y, N11Q, and A12G) to catalysis and to binding of the electrophilic substrate. All of the chimeric and mutant enzymes displayed affinity for the glutathione affinity matrix used in the purification, indicating that the glutathione-binding site was functional. GST r1(6)r8, r125)r8, and all other variant forms were catalytically active, expressed in moderate to high yields, and appeared to be stable in the pure state. However, GST r1(100)r8 was unstable and obtained in low yield. It is worth noting that the base composition in the 5' coding end strongly affected the expression levels of the proteins. For example, GST r1(25)r8 N11Q was obtained in 10-fold higher yield than GST r1(25)r8, as a result of substituting two bases in codon 11. This dependence on optimal codons at the beginning of the mRNA was utilized to increase the expression levels of GST 8–8 in a rational way. Five codons in the 5'-coding region of the subunit 8 cDNA, which are rarely present in highly biased genes in E. coli (Andersson and Kurland, 1990), were selected for silent random mutagenesis. Thus, a library of expression vectors was created with the goal of improving the protein expression level. This successful approach led to the isolation of a clone capable of expressing GST 8–8 at 25-fold increased level compared with the previously published pKGTRA8 (Stenberg et al., 1992).

N-terminal amino acid sequence analysis of GST r1(25)r8 A12G confirmed the expected primary structure SGKPVLHY-FNGRGRMEEC. Likewise, in the heterologously expressed GST 1–1, the initiator Met is removed by the E. coli Met aminopeptidase, leaving a Ser as the N-terminal residue of the recombinant protein (Wang et al., 1989). In contrast, the N-terminal Met is retained in GST 8–8 (Stenberg et al., 1992), as expected for a sequence with Glu as the penultimate amino acid residue (Dalbøge et al., 1990).

Kinetic Characterization of Chimeric GSTs—Table I shows the specificity constants (kcat/Km) observed under steady-state conditions for GSTs 1–1 and 8–8 as well as for all mutant forms with five different substrates. The substrates chosen for the analysis were those for which distinct differences in catalytic activity have been established between GSTs 1–1 and 8–8 (Mannervik and Danielson, 1988). The kcat/Km values for the mutant GST 1–1 A12G were indistinguishable from those of wild-type GST 1–1 with all substrates tested, whereas GST r1(6)r8 displayed the properties of GST 8–8. All other variant GSTs showed specificity constants toward CDNB that were similar to those of GST 8–8, which is 16-fold lower than that of GST 1–1. The catalytic efficiencies of GST r1(25)r8 and its H8Y and N11Q mutants, measured with the substrates containing α,β-unsaturated functional groups, fell between the extremes of the values for the two parental enzymes. In contrast, GST r1(25)r8 A12G, which only differs from GST r1(25)r8 in lacking a methyl group in position 12, was essentially as efficient as GST 8–8 in catalyzing Michael additions with the four α,β-unsaturated carbonyl compounds tested.

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DISCUSSION

Earlier investigations have shown that rat class Alpha GST 8–8 is much more efficient than GST 1–1 in inactivating 4-hydroxalkenals by catalyzing their conjugation with glutathione (Danielson et al., 1987). Although the sequence identity between GST 1–1 and GST 8–8 is only 59% (Fig. 1), indirect evidence for heterodimer formation has been reported (Johnson et al., 1990). Also, the overall fold is assumed to be similar for isoenzymes belonging to the same structural class. The inherent substrate diversity (GST 8–8 is more than 100-fold more active with 4-hydroxypentenal than is GST 1–1) in combination with structural similarities make GST 1–1 and GST 8–8 an ideal pair for the construction of functional chimeras with the goal of elucidating the structural basis for function.

The chimeric GST r1(25)r8, in which the N-terminal segment of subunit 1 and the remainder to the C terminus from subunit 8 are indicated by vertical arrows. The glutathione-binding (G-site) residues are indicated by g, and the second-substrate binding (H-site) residues are indicated by h below the sequences, based on comparison with the human class Alpha subunit A1 structure (Sinning et al., 1993). The exons of the corresponding genomic DNA, determined for subunit 1 (Telakowski-Hopkins et al., 1986), are shown below the sequences.

![Amino acid sequences of GST subunits 1 and 8. Positions of identity between the sequences are indicated by dashes. The initiator methionine is missing in the recombinant subunit 1, but it is retained in subunit 8. Fusion points for chimeric structures involving an N-terminal segment of subunit 1 and the remainder to the C terminus from subunit 8 are indicated by vertical arrows. The glutathione-binding (G-site) residues are indicated by g, and the second-substrate binding (H-site) residues are indicated by h below the sequences, based on comparison with the human class Alpha subunit A1 structure (Sinning et al., 1993). The exons of the corresponding genomic DNA, determined for subunit 1 (Telakowski-Hopkins et al., 1986), are shown below the sequences.](http://www.jbc.org/)

**Table I**

Kinetic parameters for GST 1–1, 8–8 and mutant forms

| GST         | CDNB* | Ethacrynic acid* | 4-Hydroxypentenal* | 4-Hydroxynonenal* | Croton aldehyde* |
|-------------|-------|------------------|--------------------|-------------------|------------------|
|             | kcat/Km |                  |                    |                   |                  |
| 1–1         | 126 ± 14 | 3 ± 1 | 2 ± 0.5 | 45 ± 4.5 | 1.6 ± 0.14 |
| 1–1 A12G    | 117 ± 11 | 3 ± 0.6 | 3 ± 0.9 | 62 ± 9.8 | 0.8 ± 0.02 |
| r1(25)r8    | 9 ± 0.5 | 33 ± 7.5 | 6 ± 1.9 | 50 ± 2.1 |                  |
| r1(25)r8 H8Y| 12 ± 0.2 | 41 ± 1.2 | 20 ± 4.8 | 194 ± 32 |                  |
| r1(25)r8 N11Q| 10 ± 0.2 | 39 ± 0.7 | 13 ± 3 | 88 ± 3.5 |                  |
| r1(25)r8 A12G| 8 ± 0.6 | 192 ± 21 | 216 ± 42 | 2440 ± 110 | 164 ± 10.5 |
| r1(6)r8     | 5 ± 0.3 | 347 ± 23 | 502 ± 122 | 2710 ± 610 |                  |
| 8–8         | 8 ± 0.6 | 353 ± 35 | 502 ± 177 | 2710 ± 320 | 189 ± 4.1 |
| k2 (M⁻¹s⁻¹) | 0.015 ± 0.0018 | 1.03 ± 0.30 | 0.94 ± 0.48 | 0.96 ± 0.48 | 0.43 ± 0.09 |

*a* Substrate.
Alignment of amino acid residues contributing to the second substrate-binding site (H-site) in class Alpha GSTs

The identification of H-site residues is based on comparison with the structure of human GST A1–1 (Sinning et al., 1993). The sequence information for the GST subunits was derived from the following sources: rat 8 (Stenberg et al., 1992), mouse A4 (Zinniak et al., 1992), human 5.8 (Singhal et al., 1994a), chicken CL3 (Chang et al., 1992), rat 1 (Pickett et al., 1984), human A1 (Board and Webb, 1987; Tu and Qian, 1986), human A2 (Rhoods et al., 1987). Dashes mark identities with rat GST subunit 8; unknown residues in the human GST 5.8 sequence are left blank.

| GST subunit | Amino acid residue | 10 | 12 | 13 | 14 | 15 | 104 | 107 | 110 | 111 | 208 | 213 | 216 | 220 | 222 |
|-------------|--------------------|----|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Rat 8       | F                  | G  | R  | G  | R  | M  | I   | G   | P   | F   | P   | V   | V   | L   | F   |
| Mouse A4    | —                  | —  | —  | —  | —  | —  | A   | V   | —   | —   | —   | —   | —   | —   | —   |
| Human 5.8   | —                  | —  | —  | —  | —  | —  | A   | V   | —   | —   | —   | —   | —   | —   | —   |
| Chicken CL3 | —                  | —  | —  | K  | G  | L  | S   | Q   | V   | I   | M   | I   | A   | F   | M   |
| Rat 1       | —                  | A  | —  | —  | —  | E  | —   | —   | —   | —   | V   | M   | L   | A   | F   |
| Human A1    | —                  | A  | —  | —  | —  | E  | L   | L   | —   | V   | M   | L   | A   | F   | —   |
| Human A2    | S                  | I  | —  | —  | —  | E  | L   | L   | —   | M   | L   | S   | F   | —   | —   |

1993), residues 18 and 25 are far removed from the active site and were therefore not mutated. However, the A12G substitution in GST r1(25)r8 essentially restored the catalytic properties of GST 8–8. The effect of the mutation was reflected in a ΔG value of 10 kJ/mol of incremental transition state stabilization (calculated from the kcat/Km values, cf. Danielsen et al., 1987) when monitored with 4-hydroxynonenal for GST r1(25)r8 A12G as compared with GST 1–1. In contrast, only 0.3 and 0.8 kJ/mol were calculated for GST 1–1 A12G and GST r1(25)r8, respectively, as compared with GST 1–1, showing that the effect of the A12G mutation is dependent on the structural context.

Based on the three-dimensional structure of human GST A1–1 (Sinning et al., 1993), which is the human enzyme most similar to rat GST 1–1, Ala in position 12 is one of the residues forming the active site. However, it is situated approximately 9 Å from the sulfer of enzyme-bound glutathione and 9 Å from the electrophilic group of the second substrate, which reacts with the sulfur in the catalyzed reaction. Assuming that the overall architecture of the active sites of GST 1–1 and GST 8–8 is similar to that of human GST A1–1, residue 12 is probably not in direct contact with either GSH or the electrophilic substrate, but mediates its effect via interactions with other structural elements that contribute to the active site. Gly allows greater variation in the conformation of the polypeptide chain than do other amino acid residues (Creighton, 1993) and may induce changes in the topology of the active site when it replaces Ala.

The second substrate binding site, or H-site, in human GST A1–1 is highly hydrophobic, a property that appears to be preserved in both GST 1–1 and GST 8–8. However, 9 of the approximately 15 amino acids that contribute to the H-site differ between GST 1–1 and GST 8–8 (Table II). A larger substrate-binding cavity in GST 8–8, corresponding to approximately five methyl groups, can be calculated from the volume enclosed by the van der Waals radii of the 9 residues differing in the H-site between subunit 1 and 8 (Creighton, 1993). However, this approximate calculation does not consider possible structural adjustments induced by the amino acid substitutions in the active site.

Comparison of class Alpha primary structures reveals that mouse GST A4–4 (Zinniak et al., 1992), human GST 5.8 (Singhal et al., 1994a), chicken GST CL3 (Chang et al., 1992), and rat GST 8–8 form a distinct subgroup within the Alpha class (Singhal et al., 1994b). For example, the amino acid sequence of rat GST 8–8 is 91% identical to that of mouse GST A4–4. All GSTs from this subgroup are highly active with 4-hydroxynonenal with some uncertainty for GST CL3, which has not been assayed with this substrate.

It is clear from the properties of the GST 1–1 Ala-12 → Gly mutant that the Ala-12 → Gly substitution alone in the GST subunit 1 framework cannot confer the high alkenal activity exhibited by GST 8–8 onto GST 1–1. Contributions by other residues, yet to be defined, are also needed. A closer examination of postulated H-site residues within the subgroup of sequence-related isoenzymes with high 4-hydroxyalkenal activity reveals that Gly-12, Pro-110, Phe-111, Val-213, Val-216, and Leu-220 are conserved, whereas other residues are variable (Table II). Further experiments are needed to clarify whether or not it would be possible to redesign the active site of GST 1–1 by substitution of a limited number of residues in the H-site to mimic the substrate profile of GST 8–8.

It is also noteworthy that the residue in position 12 is 1 out of the 11 amino acids that differ between the human isoenzymes GST A1–1 and GST A2–2 (Table II), which show divergent substrate specificities (Chow et al., 1988; Burgess et al., 1989). Furthermore, the amino acid at the topologically equivalent position in two class Mu isoenzymes has been suggested as a determinant for stereoselectivity toward α,β-unsaturated ketones (Zhang et al., 1992; Shan and Armstrong, 1994). Van Ness et al. (1994) have constructed chimeric GSTs in an attempt to identify the contribution of three different regions of a mouse enzyme to the high activity with the hepatocarcinogen aflatoxin B1, 8,9-epoxide. However, the specific residues of importance for the substrate selectivity were not identified.

This investigation complements previous studies showing that construction of chimeric GSTs is a feasible approach to modular design of new functional entities (Zhang and Armstrong, 1990; Mannervik et al., 1990; Zhang et al., 1992; Björnstedt et al., 1992; Van Ness et al., 1994). The positive outcome of these experiments lends support to the proposal that recombination of segments of genomic DNA may have been an evolutionary mechanism for generation of novel GST isoenzymes (Mannervik, 1985). The chimera r1(25)r8 corresponds to the product that would result from a switching from the gene for subunit 1 to the gene for subunit 8 in a position between exons 2 and 3 (residues 26–29 are identical between the subunits, cf. Fig. 1).

GSTs are capable of interacting with a very broad range of molecular structures (Mannervik and Danielson, 1988) and have attractive features for protein engineering, including high yield expression in E. coli and lack of post-translational modifications. It should be possible to redesign GSTs to obtain catalysts for GSH conjugation of a very broad range of electrophilic compounds by optimizing the active site structure of the enzyme for the particular reaction to be catalyzed. This has recently been attempted via selection of GST mutants by use of phage display (Widersten and Mannervik, 1995; Mannervik et al., 1995). Conventional wisdom in the field of detoxication enzymes maintains that such enzymes have low catalytic efficiencies in order to be able to react with a broad range of substrates (Jakoby, 1980). According to this paradigm, it would appear difficult to obtain mutant enzymes with high catalytic turnover. However, the present work shows that high catalytic...
efficiency can be obtained by a single point mutation to give a variant GST, i.e., r1(25)/8 Ala-12 → Gly, with high catalytic efficiency not only in relative terms, but also on an absolute scale (kcat/Km > 10⁶ M⁻¹ s⁻¹).

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