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# Effects of directed mutagenesis on conserved arginine residues in a human Class Alpha glutathione transferase

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Glutathione transferase (GST) $\epsilon$ (also known as GST2 or GST $B_1B_1$), the major Class Alpha GST in human liver has been subjected to oligonucleotide-directed site-specific mutagenesis. Four arginine residues, R13, R20, R69 and R187, of which all but R69 are strictly conserved through GST Classes Alpha, Mu and Pi have been replaced by Ala. The mutant enzymes have been expressed in *Escherichia coli*, purified by affinity chromatography and characterised. Compared with the wild-type enzyme, all mutant GSTs had altered catalytic properties. All mutants had decreased specific activity with 1-chloro-2,4-dinitrobenzene (CDNB). Mutants R13A, R69A and R187A also showed decreased activities with other substrates such as cumene hydroperoxide (CuOOH) and androstenedione. In contrast, mutant R20A had an increased peroxidase activity and an isomerase activity essentially the same as that of the wild-type GST. With the substrates used, $k_{cat.}/K_m$ values were decreased for all mutant GSTs. Increases in the $[S_{0.5}]$ values were most significant for glutathione (GSH), while values for CDNB and CuOOH were less markedly affected. Thus, various kinetic data indicate that the GSH affinity has been reduced by the mutations and that this loss of affinity is linked to the decreased specific activities. Inhibition studies showed an increased sensitivity towards *S*-hexyl-GSH; this was particularly marked for mutant R69A. Mutant R20A had a lowered $[I_{50}]$ value but, in contrast, also the highest $[I_{80}]$ value as compared with the wild-type enzyme. Towards bromosulphophthalein, mutants R20A and R69A had a markedly increased sensitivity, about 35-fold in comparison with the wild-type. The inhibition properties of mutant R187A were similar to those of the wild-type enzyme and the properties of mutant R13A were in between. The increased sensitivity to *S*-hexyl-GSH, in contrast with the decreased affinity for GSH, was suggested to be due to an altered distribution between conformational states of the enzyme induced by the mutations. The arginine residues in positions 13, 20 and 69 all seem to be important for the catalytic properties of GST. Further, the inhibition studies indicate a role of arginine residues in the stabilisation of conformational states of the enzyme.

## INTRODUCTION

Glutathione transferases (GSTs) have been studied extensively, and a large number of primary structures have been determined by protein and DNA sequence analysis (Mannervik & Danielson, 1988). The three-dimensional structure has been probed by X-ray-diffraction analysis of representatives of mammalian GSTs of Classes Alpha, Mu, and Pi (Sesay *et al.*, 1987; Schäffer *et al.*, 1988; Cowan *et al.*, 1989). Chemical-modification studies have been made in order to identify amino acid residues essential for catalytic activity. In spite of these efforts, the chemical groups involved in the catalytic function remain unknown for all of the GSTs investigated. Definitive information has to await the results of X-ray crystallography, but it may be predicted that the final analysis will require the complement of site-directed mutagenesis in order to evaluate the significance of each individual amino acid. In the present investigation the latter approach has been initiated for the human Class Alpha GST $\epsilon$, also known as GST2 or GST $B_1B_1$, with the aim of exploring the potential role of amino acids that have been conserved in the evolution of the sequences of the various GSTs.

## MATERIALS AND METHODS

Plasmid pTacGST2 (Board & Pierce, 1987) containing the cDNA for the wild-type enzyme GST2 was used for expression of the protein. When purified from human sources, the isoenzyme corresponding to the protein expressed by this construct has variously been termed GST2 type 1, GST $\epsilon$ or $B_1B_1$. Plasmid pUC 18 (Vieira & Messing, 1982) and bacteriophages M13mp18 and M13mp19 (Norrander *et al.*, 1983) were obtained from Boehringer-Mannheim. Oligonucleotide-directed *in vitro* mutagenesis kit and [$^{32}$P]dCTP were from Amersham International. Klenow fragment of DNA polymerase I and restriction enzymes were from Amersham International, Boehringer-Mannheim, Promega and Pharmacia LKB Biotechnology. Mutagenic oligonucleotides and sequencing primers were obtained from the Department of Immunology, Biomedical Centre, Uppsala, Sweden.

Sephadex G-25 and epoxy-activated Sepharose 6B were from Pharmacia LKB Biotechnology, Uppsala, Sweden. *S*-Hexyl-GSH was synthesized according to method A of Vince *et al.* (1971).

Isopropyl $\beta$-D-thiogalactoside (IPTG), ampicillin, GSH and other chemicals were of highest quality available from commercial sources. $\Delta^5$-Androstene-3,17-dione was generously given by Dr. Paul Talalay, Johns Hopkins University, Baltimore, MD, U.S.A.

### Construction of mutagenesis vector

In order to construct a vector for the production of single-stranded cDNA for site-directed mutagenesis, the GST2 cDNA and the *tac* promoter were cleaved from the pTacGST2 plasmid (Board & Pierce, 1987) by partial *ScaI* and *Hin*dIII digestions

Abbreviations used: BSP, bromosulphophthalein; CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; DTT, dithiothreitol; GST, glutathione transferase; IPTG, isopropyl $\beta$-D-thiogalactoside; $[I_{50}]$ and $[I_{80}]$ are concentrations of inhibitor giving 50% and 80% inhibition of activity respectively.

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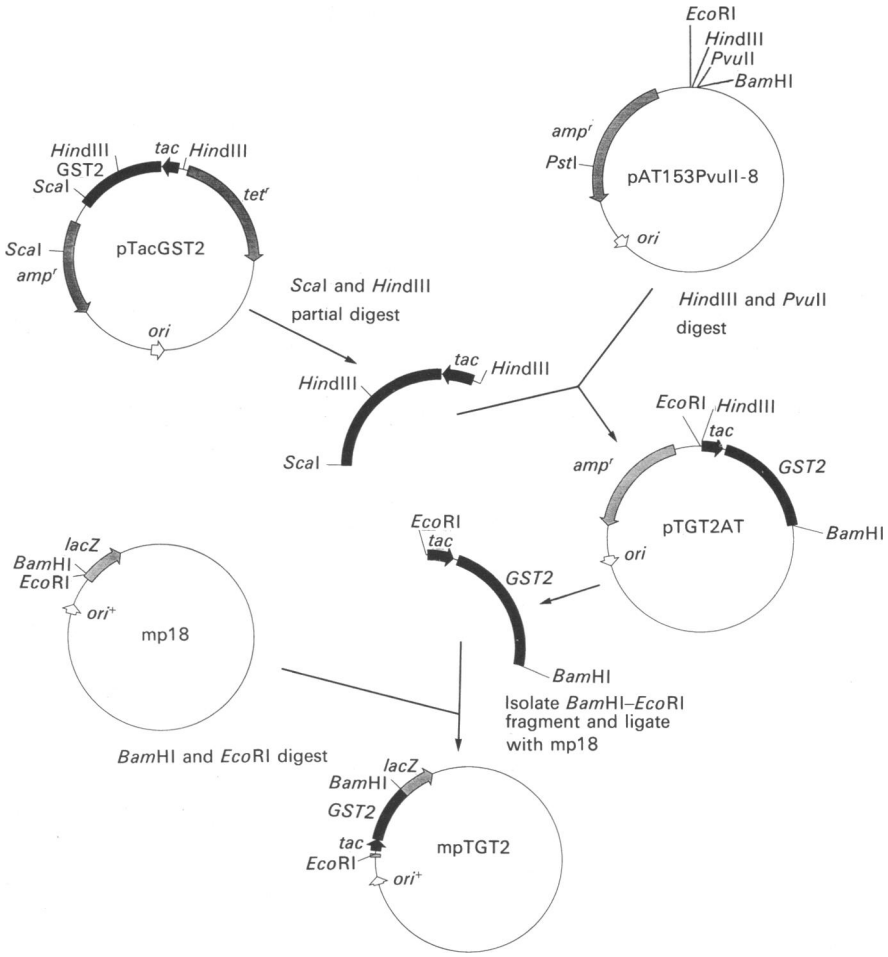


**Fig. 1. Schematic diagram of the steps taken to construct a movable cassette containing the *tac* promoter and the GST2 cDNA**

The cassette was cloned into M13mp18 to provide single-stranded DNA for oligonucleotide-directed mutagenesis. The procedure is described in the text. ■ Indicates the position of the GST2 cDNA. Abbreviations: *tac*, *tac* promoter; *amp*$^r$, ampicillin-resistance gene; *tet*$^r$, tetracycline-resistance gene; ori, origin of replication; *Hin*dIII etc. are restriction endonucleases.

and the sequence encoding GST was isolated and ligated into the *Pvu*II and *Hin*dIII sites of the plasmid pAT153PvuII-8 (Gianneli *et al.*, 1983). The resulting plasmid, termed pTGT2AT, was digested with *Eco*R1 and *Bam*H1 and the cassette containing the GST2 cDNA and the *tac* promoter was ligated into the *Eco*R1 and *Bam*H1 sites of M13mp18 (Norrander *et al.*, 1983). The resulting M13 phage was termed mpTGT2 (Fig. 1). *Escherichia coli* JM103 infected with mpTGT2 were capable of expressing GST2 when grown in the presence of 0.1 mM-IPTG.

### Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis was carried out with an Amersham kit according to the manufacturer's instructions. Clones with the required mutation were identified by sequencing using the chain-termination method of Sanger *et al.* (1977). To express the modified protein, the cassette containing the mutated cDNA and the *tac* promoter was excised from the M13 replicative form with *Bam*HI and *Eco*RI and ligated between the corresponding restriction sites in the vector pUC18.

### Enzyme production and purification

Human wild-type and mutant GSTs were expressed in *E. coli* strains JM103 and JM109 respectively. An overnight culture containing plasmid was sub-cultured at a dilution of 1:100 in LB-broth (Maniatis *et al.*, 1982) supplemented with ampicillin, (50 $\mu$g/ml) at 37 °C with vigorous shaking. When the $A_{600}$ reached a value of 0.3, IPTG was added to a final concentration of 1 mM, and the culture was allowed to grow overnight for expression of GST2. The following day, cells were harvested by centrifugation at 7000 *g* for 10 min and washed in 50 mM-Tris/HCl (pH 7.8) containing 150 mM-NaCl and then resuspended in the same buffer in 1/30 of the original volume. Lysozyme was added at 10 mg/l of original culture volume and left for 1 h. The cell suspension was sonicated with a Branson model L sonifier at maximal power output and centrifuged at 25 000 *g* for 20 min. The sediment was suspended in 50 mM-Tris/HCl (pH 7.8), containing 150 mM-NaCl, sonicated and centrifuged; this procedure was repeated once. The combined supernatant fractions were run through a Sephadex G-25 column equilibrated with 10 mM-Tris/HCl, pH 7.8, containing 1 mM-2-mercaptoethanol. Enzymes were then purified by affinity chromatography on an *S*-hexyl-GSH matrix (Mannervik & Guthenberg, 1981) followed by dialysis in the same buffer as above. All steps were performed at 4 °C.

Homogeneity of the purified GSTs was checked by SDS/PAGE (Laemmli, 1970). Non-denaturing agarose-gel electrophoresis (Board & Pierce, 1987) was used to verify any expected changes in the net charge of the mutant enzymes; after electrophoresis, the proteins were capillary-blotted on to nitrocellulose and detected by an e.l.i.s.a. (Board, 1984). The 'wild-type' GST used was the recombinant protein, which appears indistinguish-

able in catalytic properties from the enzyme isolated from human liver (Board & Pierce, 1987; K. Berhane & B. Mannervik, unpublished work).

### Characterization of the enzymes

Specific activities of the purified enzymes were determined in three different assay systems. A conjugation reaction was measured spectrophotometrically at 340 nm with 1 mM-CDNB, 5% (v/v) ethanol (solvent for CDNB), and 1 mM-GSH in 0.1 M-sodium phosphate containing 1 mM-EDTA, pH 6.5 at 30 °C (Habig & Jakoby, 1981). A reduction reaction was monitored spectrophotometrically at 340 nm with 1.5 mM-CuOOH, 5% (v/v) ethanol (solvent for hydroperoxide), 1 mM-GSH, 0.3 unit of glutathione reductase and 0.1 M-sodium phosphate, pH 7.0, containing 1 mM-EDTA at 30 °C (Lawrence & Burk, 1976). An isomerization reaction was measured with 0.068 mM-$\Delta^5$-androstene-3,17-dione, 2% (v/v) methanol (solvent for steroid), 0.1 mM-GSH and 0.1 mM-DTT in 0.025 M-Tris/potassium phosphate, pH 8.5. The reaction was monitored spectrophotometrically at 248 nm (Benson *et al.*, 1977).

The enzymes were diluted in 10 mM-Tris/HCl/1 mM-EDTA, pH 7.8, containing 20% (v/v) glycerol to maintain enzymic activity. All assays were done in the presence of a final concentration of 1% (v/v) glycerol.

Reactions were started by addition of enzyme, except for the reaction involving GSH and CDNB, in which the enzyme was pre-incubated with GSH and the reaction started by addition of CDNB.

### Determination of kinetic constants

The parameter $k_{cat.}/K_m$ was determined as $v/[S][E]_{tot.}$ under first-order conditions (low concentration) with respect to the varied substrate; for GSH with a fixed concentration of 1 mM-CDNB, for CDNB and for CuOOH with a fixed concentration of 1 mM-GSH. Other experimental conditions were the same as for determination of specific activities.

Initial-rate data showed strong deviations from Michaelis–Menten kinetics, but maximal velocities ($V_{max.}$) could be estimated by extrapolation to infinite substrate concentrations. Conventional $K_m$ values could not be determined and, in order to get some information about substrate 'affinities', $[S_{0.5}]$, defined as the substrate concentration giving half-maximal velocity, was estimated from $v$-versus[S] plots at high concentrations (close to saturating) of the non-varied substrate.

### Inhibition studies

Two inhibitors, *S*-hexyl-GSH and BSP, were chosen for comparisons of the wild-type with the mutant enzymes. $[I_{50}]$ and $[I_{80}]$ are defined as the inhibitor concentrations that afford 50% and 80% inhibition of the enzyme activity measured with CDNB as electrophilic substrate in the standard assay system. The inhibitor was added to the ongoing reaction 2 min from the start, when initial-rate conditions still obtain. This order of addition was chosen in order to get an internal standard for every measurement and was found to give the most reproducible results. However, it should be noted that this procedure gives $[I_{50}]$ (and $[I_{80}]$) values that are significantly lower than the corresponding values obtained when the inhibitor was present from the beginning of the enzymic reaction.

## RESULTS

### Construction of mutant forms of GSTs

Four arginine residues, in positions 13, 20, 69 and 187, three of which are strictly conserved in the known mammalian GSTs, were selected for oligonucleotide-directed mutagenesis. Table 1 lists the oligonucleotides used to convert the arginine residues into alanine residues. In all cases, the entire cDNA encoding GST2 was sequenced in order to verify that the desired mutation had been obtained and that no other alterations of the nucleotide sequence had occurred. The selection of clones of mutated DNA was made by sequencing a limited number (four to six) of isolates after mutagenesis. In each experiment more than half of the isolates contained the desired sequence.

Mutant enzyme forms were expressed in bacteria harbouring plasmids with the corresponding genes. After lysis of the cells, effected by lysozyme treatment followed by sonication, GST was isolated by affinity chromatography on immobilized *S*-hexyl-GSH. It was found that the yield of purified wild-type enzyme was 30–50-fold higher than those of mutants R13A, R69A and R187A and 4-fold higher than for R20A. In order to probe the binding properties of the mutant enzymes, chromatography was also performed on immobilized GSH (Simons & Vander Jagt, 1977). It was noted that a significantly larger fraction of the total enzyme activity was not bound to the different affinity matrices when the mutant forms were used. Poor binding affinity was particularly obvious for mutant R13A.

### Characterization of the mutant enzymes

In most respects, the physico-chemical properties of the mutated forms of GST were similar to those of the wild-type enzyme. The apparent subunit $M_r$ was indistinguishable from that of the wild-type enzyme as estimated by SDS/PAGE. All enzyme forms appeared to be dimers, as judged by the elution volumes determined by gel filtration on Sephacryl S-300 (results not shown). The expected charge differences introduced by the mutations were reflected in the electrophoretic mobilities of the mutant proteins observed by gel electrophoresis under non-denaturing conditions (Fig. 2). However, it should be noted that although all arginine→alanine mutations formally invoke a change of charge of 1 unit, this is not reflected in a uniform decrease in electrophoretic mobility. The observed differences may be related to the degree of solvent accessibility of the mutated residue or to altered conformational states of the protein induced by the mutations. Nevertheless, the results confirm the mutations established at the DNA level as being expressed at the protein level, and show that any altered properties of the mutant enzymes can be related to the structure of individual subunits rather than to alterations of the quaternary structure of the proteins.

In the case of mutant R13A, the *N*-terminal amino acid sequence was determined on a gas-phase sequenator and found to be AEKPKLHYFNAAGRM, confirming the substitution of alanine for arginine in position 13 of the sequence. The cor-

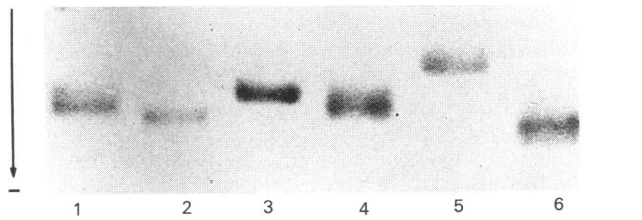


**Fig. 2. Non-denaturing agarose electrophoresis of wild-type and mutant GSTs**

Lane 1, mutant R187A; lane 2, mutant R69A; lane 3, mutant R13A; lane 4, mutant R20A; lane 5, mutant K36E; lane 6, wild-type GST2. The mutant in lane 5, K36E, involves twice the charge change as compared with other mutations and is included for reference.

**Table 1. Sites in the primary structure of human GST2 selected for mutation and definition of the mutant forms of the enzymes**

Arg residues in the segments of amino acid sequence given were mutated into Ala residues by use of the oligodeoxynucleotides shown in the bottom line. The codon to be changed in the wild-type enzyme is written in the middle and mismatches are indicated by asterisks.

| Mutant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| R13A | | F | N | A | $R_{13}$ | G | R | M | |
| | 5'- | TTC | AAT | GCA | CGG | GGC | AGA | ATG | -3' |
| | | | | | * * * | | | | |
| | 3'- | AAG | TTA | CGT | CGA | CCG | TCT | TAC | -5' |
| R20A | | E | S | T | $R_{20}$ | W | L | L | |
| | 5'- | GAG | TCC | ACC | CGG | TGG | CTC | CTG | -3' |
| | | | | | * * * | | | | |
| | 3'- | CTC | AGG | TGG | CGA | ACC | GAG | GAC | -5' |
| R69A | | V | Q | T | $R_{69}$ | A | I | L | |
| | 5'- | GTG | CAG | ACC | AGA | GCC | ATT | CTC | -3' |
| | | | | | * * | | | | |
| | 3'- | CAC | GTC | TGG | CGT | CGG | TAA | GAG | -5' |
| R187A | | L | K | T | $R_{187}$ | I | S | N | |
| | 5'- | CTG | AAA | ACC | AGA | ATC | AGC | AAC | -3' |
| | | | | | * * | | | | |
| | 3'- | GAC | TTT | TGG | CGT | TAG | TCG | TT | -5' |

**Table 2. Specific activities and $k_{cat.}/K_m$ values**

Specific activities and $k_{cat.}/K_m$ were determined for three substrates in different assay systems as described in the Materials and methods section. Values are means ± S.D., generally based on $n \geq 5$.

| | | Specific activity ($\mu$mol·min$^{-1}$·mg$^{-1}$) | | | $k_{cat.}/K_m$ (mM$^{-1}$·s$^{-1}$) (% of wild-type value) | | |
|---|---|---|---|---|---|---|---|
| GST mutant | Substrate... | CDNB | CuOOH | Andro-stenedione | GSH | CDNB | CuOOH |
| Wild-type | | 60.2±3.0 | 10.2±0.5 | 12.1±0.8 | 260.4±17.7 (100) | 99.8±4.7 (100) | 34.7±0 (100) |
| R13A | | 7.9±0.9 | 3.0±0.2 | 1.5±0.1 | 11.9±0.6 (5) | 5.8±1.0 (6) | 5.6±1.1 (17) |
| R20A | | 8.9±0.8 | 19.7±1.4 | 9.7±0.6 | 28.7±1.1 (11) | 15.4±0.6 (15) | 30.9±4.7 (89) |
| R69A | | 15.4±0.8 | 7.2±1.8 | 1.7±0.2 | 18.1±2.5 (7) | 9.9±1.6 (10) | 15.2±2.5 (43) |
| R187A | | 32.3±1.1 | 6.3±0.6 | 3.8±0.2 | 154.5±10.2 (59) | 55.2±7.5 (55) | 22.6±3.2 (65) |

responding wild-type sequence was determined for comparison; in both structures the initiator methionine (position 1) had been eliminated.

### Kinetic parameters

Specific activities with three distinct types of substrates, CDNB, CuOOH and $\Delta^5$-androstene-3,17-dione, were determined for the mutant enzymes (Table 2). The activity with CDNB was lower for all mutant forms than for the wild-type enzyme. Mutants R187A, R69A, and R13A all showed significantly lower peroxidase and isomerase activities, as measured with CuOOH and androstenedione, respectively. Mutant R20A displayed a twofold higher peroxidase activity, whereas the isomerase activity was not significantly different from that of the wild-type enzyme.

The kinetics of GSTs are known to be complex, requiring rate equations with many parameters for full description of the rate behaviour (see Mannervik, 1985). Meaningful comparisons of individual parameters cannot be made, since the individual parameter values of a complex model are not independent, but linked to one another through the variance-co-variance matrix (Mannervik, 1982). However, the kinetics at low concentrations of electrophilic substrate and a high concentration of GSH can be approximated by the Michaelis–Menten equation, and $k_{cat.}/K_m$ can be estimated by measurements at low substrate concentrations (cf. Danielson & Mannervik, 1985). This estimate of catalytic efficiency is a physiologically relevant parameter, since the enzyme can be expected to encounter only low concentrations of the toxic second substrate in the cell. The $k_{cat.}/K_m$ values determined (Table 2) show a similar relative rank order to the specific activities, the main difference being that for mutant R20A the specific activity with CuOOH was 200%, whereas the $k_{cat.}/K_m$ value was 89% of the wild-type values.

In spite of the kinetic complexities, additional kinetic parameters were determined for further characterization of the different mutant forms. Maximal velocity ($V_{max.}$) and substrate concentration giving half-maximal velocity, [$S_{0.5}$], were estimated for the different enzymes at close-to-saturating concentrations of the non-varied substrates (Table 3). The actual curve shapes differed from enzyme to enzyme, and the parameters are not directly comparable in terms of elementary rate constants and concentrations of non-varied substrates, but the $V_{max.}$ and [$S_{0.5}$] values still express catalytic potential and a measure of 'apparent

**Table 3. Enzymic rate-saturation parameters**

$V_{max.}$ is the maximal velocity estimated from the initial rate versus substrate concentration curve. $[S_{0.5}]$ is defined as the substrate concentration giving half-maximal velocity, as estimated from the same curve. Concentrations were 0.01–12 mM for GSH, 0.01–2.5 mM for CDNB and 0.008–3 mM for CuOOH. Other assay conditions were as described in the Materials and methods section

| | $[S_{0.5}]$ (mM) | | | $V_{max.}$ ($\mu$mol·min$^{-1}$·mg$^{-1}$) | | |
|---|---|---|---|---|---|---|
| GST mutant | GSH | CDNB | CuOOH | GSH | CDNB | CuOOH |
| Wild-type | 0.1 | 0.5 | 0.3 | 75 | 75 | 14 |
| R13A | 0.8 | 0.7 | 0.5 | 12 | 9 | 5 |
| R20A | 0.6 | 0.5 | 0.4 | 14 | 11 | 20 |

**Table 4. Inhibition parameters**

$[I_{50}]$ and $[I_{80}]$ are the concentrations of inhibitor giving 50 and 80% inhibition of activity respectively, as measured at pH 6.5 with 1 mM-GSH and 1 mM-CDNB in 0.1 M-sodium phosphate containing 1 mM-EDTA.

| | [*S*-Hexyl-GSH] ($\mu$M) | | [BSP] ($\mu$M) |
|---|---|---|---|
| GST mutant | $[I_{50}]$ | $[I_{80}]$ | $[I_{50}]$ |
| Wild-type | 0.7 | 4.5 | 7 |
| R13A | 0.4 | 5.6 | 3 |
| R20A | 0.1 | 7.5 | 0.2 |
| R69A | 0.03 | 2.0 | 0.2 |
| R187A | 0.4 | 2.8 | 7 |

substrate affinity', even though there is no obvious relationship between these parameters and $k_{cat.}/K_m$. Using a simplistic interpretation, mutants R13A and R20A display a decreased binding affinity for GSH, as judged by the elevated $[S_{0.5}]$ values. Corresponding values for CDNB and CuOOH remain essentially unchanged. The relative $V_{max.}$ values mirror the corresponding specific activities (cf. Table 2).

The initial part of the progress curve of the reaction catalysed by GST was not linear if the reaction was initiated by addition of enzyme. However, pre-incubation of GST with GSH, followed by initiation of the reaction by addition of CDNB, afforded essentially zero-order kinetics for the wild-type enzyme as well as for mutant forms R13A and R17A in the standard assay system, using 0.1–1 $\mu$g of enzyme. However, mutant forms R20A and R69A required 5–10-fold increased concentrations of GSH (5–10 mM) to overcome the non-linearity in the progress curves. A similar curvature in the progress curve was noted for mutant forms R20A and R69A when recording the isomerase activity, especially with mutant form R69A, which has a low specific activity with androstenedione.

### Inhibition studies

Inhibition parameters were determined in the standard assay system using CDNB as the electrophilic substrate. Two inhibitors were investigated, *S*-hexylglutathione and BSP. In the case of *S*-hexylglutathione, two parameters, $[I_{50}]$ and $[I_{80}]$, were used, since the curve shape of residual activity versus inhibitor concentration was altered significantly for some of the mutant forms as compared with the inhibition curve for the wild-type enzyme. With this inhibitor it was found that mutant forms R69A and R20A had significantly increased affinities for the inhibitor as judged from the $[I_{50}]$ values, which reflect the low concentration range of the inhibitor (Table 4). At higher inhibitor concentrations, the difference in inhibitor strength was small as reflected in the $[I_{80}]$ values. In the case of BSP, mutant forms R20A and R69A showed significantly increased sensitivities.

## DISCUSSION

In the search for amino acid residues of functional importance, the arginine residues that are conserved in all known amino acid sequences in the three classes of mammalian cytosolic GST (Mannervik & Danielson, 1988) were investigated. These residues, Arg-13, Arg-20 and Arg-187, belong to the fraction of approx. 5% that is conserved out of the approx. 220 residues in the mammalian GSTs. Thus they are likely to be important for the structure or the function of the enzymes. In addition, Arg-69, which is part of the conspicuous amino acid sequence 'TRAIL' that is conserved in the Class Alpha GSTs, was chosen for mutagenesis. This residue may have a counterpart in an arginine residue following a similar 'SNAIL' sequence in the corresponding position in several Class Mu and Class Pi enzymes (Mannervik & Danielson, 1988).

In general, the mutant enzymes were expressed from plasmids in *E. coli* in lower yields than the wild-type enzyme. Among the mutant forms R20A was obtained in a 10-fold higher amount than the other mutant forms. The differences in yields are not primarily related to the differences in binding to the affinity column, since different levels of activity were detected in the crude bacterial lysate. No definitive explanation can be given, but factors that might contribute to the lower yields may include decreased stability or translatability of the corresponding mRNA or decreased stability of the mutant proteins. Even though the mutant forms of GST were expressed in quantities significantly lower than that of the parental wild-type enzyme, the yields were sufficient for structure–function studies.

The physico-chemical properties of the mutant forms of GST were similar to those of the wild-type enzyme, with the exception of the difference in charge caused by substitution of alanine for arginine in the proteins. The *N*-terminal amino acid sequences of the wild-type enzyme and mutant form R13A were determined and were found to agree with the predictions based on the DNA sequences, with the exception that the initiator methionine had been eliminated in the recombinant proteins. Thus it would appear that any change in functional properties caused by the different mutations can be ascribed to the amino acid substitution *per se* and not to secondary effects caused by alteration of the overall structural properties of the enzyme.

Mutant form R13A appears to have a decreased affinity for GSH as compared with the wild-type enzyme. This conclusion is based on the decreased affinity for immobilized GSH, the eightfold increased $[S_{0.5}]$ value for GSH (Table 3) and the decreased $k_{cat.}/K_m$ value (Table 2). Using $k_{cat.}/K_m$ as a measure of the binding affinity to the transition state, it can be calculated (see Fersht, 1985) that removal of the guanidium group of arginine corresponds to a loss in binding affinity of 7.5 kJ (1.8 kcal)/mol.

Mutant R20A also exhibits decreased binding affinity for GSH, as indicated by the lowered $k_{cat.}/K_m$ value (Table 2) and increased $[S_{0.5}]$ value (Table 3) for GSH. It was previously noted that the initial non-linear phase of the progress curve of the reaction catalysed by the wild-type enzyme could be eliminated by pre-incubation of the enzyme with GSH before initiating the reaction by addition of CDNB. In the case of mutant form R20A, as well as for R69A, higher concentrations of GSH (5–10 mM) were

required to eliminate the curvature. This finding suggests that the latter mutations affect the binding of GSH, which in turn influences the distribution of the enzyme between different conformational states with different catalytic activities. Evidence for such functionally relevant conformational transitions has previously been obtained (Principato *et al.*, 1988). Consequently, Arg-20 and Arg-69 both appear to be linked to GSH binding. However, it should be noted that such effects may be indirect and could be related to in destabilization of certain conformational states of the protein rather than to direct interaction with, for example, a carboxyl group of the GSH molecule.

In the case of mutant form R20A, a 2-fold increase in specific activity for CuOOH as well as a similar increase in the $V_{max.}$ value were consistently noted. The increased apparent $V_{max.}$ value was mainly attributable to lack of inhibition by excess CuOOH in the case of mutant form R20A; the wild-type enzyme as well as mutant form R13A were both found to display substrate inhibition when CuOOH served as substrate. However, it is not clear why the mutation R20A should eliminate the inhibitory part of the rate saturation curve.

Mutant form R187A differed less from the wild-type enzyme in its catalytic properties than did any of the other arginine-mutated enzymes studied. The effects of the mutation on the kinetic properties of mutant form R187A could not be specifically related to binding of GSH or any of the second substrates investigated. It seems likely that a structural effect on the protein conformation may explain the moderate changes of the kinetic parameters observed.

The inhibition data obtained with *S*-hexyl-GSH showed that in spite of the fact that other parameters suggested that mutant forms R13A, R20A and R69A had lower binding affinity than the wild-type enzyme, the mutant [$I_{50}$] values were actually lower than that obtained for a wild-type enzyme. In particular, mutant form R69A gave a 23-fold lower value, indicating a significant increase in binding affinity for the inhibitor. These results can be rationalized by the explanation that the binding of *S*-hexyl-GSH is dominated by the hexyl substituent and not by the peptide moiety of GSH. The marked decrease in [$I_{50}$] value for mutant form R69A might indicate that the hexyl group of the inhibitor binds in the vicinity of Arg-69, since removal of the charged side chain would be expected to augment the binding of the non-polar residue of the inhibitor. However, it should also be noted that the shape of the inhibition curves was altered for most of the mutant enzymes. It can be shown that, for a simple linear inhibitor, $[I_{80}]/[I_{50}] = 4$ (Tahir & Mannervik, 1986). The wild-type enzyme and mutant form R187A give values not too far from the theoretical value, but the $[I_{80}/I_{50}]$ value for mutant forms R20A and R69A is increased to approx. 70. This change of the curve shape shows that higher-degree terms in inhibitor concentration have to be included in the rate equation, and is consistent with a reaction mechanism in which the inhibitor may bind with different affinities to distinct conformational states of the protein. Several lines of evidence suggest the existence of different conformational states of the enzyme (Mannervik & Danielson, 1988). The marked differences in inhibition parameters obtained when the inhibitor was added before as compared with after the substrates (noted in the Materials and methods section) demonstrate differences in affinity of 'resting' as compared with catalytically functioning enzyme. The [$I_{50}$] values obtained with BSP may also be explained by the assumption that mutations R20A and R69A have effected the stabilization of a conformation of the protein that has higher affinity for the inhibitor. It should be noted that the [$I_{80}$] value was not changed dramatically by any of the mutations. This parameter might reflect the binding affinity for a lower-affinity conformation of the protein. This interpretation could explain why removal of a positive charge of the protein could enhance the binding affinity of the negatively-charged BSP molecule.

The amino acid residues in GST that are involved in the binding of substrates and inhibitors are unknown. However, photo-affinity labelling of rat GST 1–1 and 1–2 with *S*-(*p*-azidophenacyl)-GSH suggests that an amino acid sequence close to the *C*-terminal contributes to the binding of the *S*-substituent of glutathione derivatives (Hoesch & Boyer, 1989). On the other hand, photo-affinity labelling with BSP of rat GST sub-unit 1 and human GST2 suggests that this inhibitor interacts with the *N*-terminal amino acid portion (Bhargava & Dasgupta, 1988). It is not obvious how the results of the present investigation may be reconciled with these observations, even though BSP may bind to a location distinct from the active site. However, arginine residues have previously been implicated in binding sites for the GSH molecule in GSTs as well as in other GSH-linked enzymes (Mannervik *et al.*, 1989). The results of the present investigation suggest that the conserved Arg residues in positions 13, 20 and 69 may contribute to the binding affinity of GSH. One function of the arginine residues may be the neutralization of the negative charge of the carboxylate groups of GSH. The importance of these carboxylate groups for binding has been demonstrated by use of GSH analogues (Adang *et al.*, 1988, 1989). Another function may be to stabilize one of the conformational states proposed for the enzyme. A shift in the relative distribution of different conformations of the protein may influence the binding of GSH and other ligands.

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