Cloning and heterologous expression of cDNA encoding class Alpha rat glutathione transferase 8–8, an enzyme with high catalytic activity towards genotoxic α,β -unsaturated carbonyl compounds

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A cDNA clone, λ GTRA8, encoding rat glutathione transferase subunit 8 has been isolated from a λ gt10 rat hepatoma cDNA library. The previously known amino acid sequence of the enzyme was used to design primers for a polymerase chain reaction that yielded a 0.3 kb DNA fragment from the hepatoma library. The 0.3 kb fragment was used as a probe for screening and a 0.9 kb cDNA clone containing a complete open reading frame was obtained. After DNA sequencing and subcloning into an expression vector, the enzyme was expressed in *Escherichia coli* and purified. Specific activities and k_{cat}/K_m values were determined for a number of substrates, including α,β -unsaturated carbonyl compounds. The highest activity was obtained with 4-hydroxyalkenals and with acrolein, genotoxic products of lipid peroxidation. In addition, the rat class Alpha glutathione transferase 8–8 displays high catalytic activity in the reaction between glutathione and the diuretic drug ethacrynic acid, a compound normally considered as a substrate characteristic for class Pi glutathione transferases.

INTRODUCTION

In the multitude of glutathione transferases (GSTs) so far characterized (Mannervik & Danielson, 1988) the rat class Alpha GST 8–8, also known as GST K (Hayes, 1986), has received special attention in view of its uniquely high catalytic efficiency with 4-hydroxyalkenal substrates (Jensson *et al.*, 1986). The k_{cat}/K_m values for long-chain 4-hydroxyalkenals exceed $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Danielson *et al.*, 1987), values that are higher by several orders of magnitude than almost all other substrates tried for any of the known GSTs. GST 8–8 has a broad tissue distribution (Hayes & Mantle, 1986; Meyer *et al.*, 1989) and even if the absolute concentration in liver is small in comparison with the major enzyme forms (Ålin *et al.*, 1989), it is noteworthy that the enzyme is the dominating form in rat erythrocytes (Dirr & Schabott, 1987; Meyer *et al.*, 1989).

cDNA and genomic DNA clones have been isolated for a large number of GSTs (see Pickett & Lu, 1989), and it was considered of interest to identify and investigate cDNA corresponding to GST 8–8 in order to obtain further insights into the evolution of its special catalytic properties within the gene superfamily (Mannervik *et al.*, 1985). The present paper describes the isolation of a cDNA clone containing the entire coding sequence for rat GST 8–8, subcloning of the cDNA in an expression vector and characterization of the recombinant enzyme.

MATERIALS AND METHODS

Materials

A rat hepatoma λ gt10 cDNA library was prepared as pre-

viously described (Pemble *et al.*, 1986). Restriction enzymes and other enzymes used in the molecular cloning were from Promega Corp. (Madison, WI, U.S.A.), Boehringer Mannheim (Mannheim, Germany), Amersham International (Amersham, Bucks., U.K.) and Perkin–Elmer, Cetus Instruments (Norwalk, CT, U.S.A.). Nucleotides were from Pharmacia LKB Biotechnology (Uppsala, Sweden). Δ^5 -Androstene-3,17-dione was generously given by Dr. Paul Talalay, The Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A. Linoleic acid hydroperoxide was kindly provided by Dr. Ralf Morgenstern, Karolinska Institute, Stockholm, Sweden, and base propenals by Dr. John W. Kozarich, University of Maryland, College Park, MD, U.S.A. All other reagents used were of the highest grade commercially available.

Preparation of cDNA for PCR

First, $2 \mu\text{l}$ (10^{11} plaque forming units/ml) of a rat hepatoma cDNA library was treated with RNAase A and DNAase I at 37°C for 30 min. Phages were precipitated with an equal volume of 20% (w/v) poly(ethylene glycol)/2 M-NaCl on ice for 1 h. After phenol/chloroform extraction, the DNA was precipitated with 1 vol. of propan-2-ol, and dissolved in water. The PCR was carried out in a total volume of $100 \mu\text{l}$ containing 10 mM-Tris/HCl, pH 8.3, 50 mM-KCl, 1 mM-2-mercaptoethanol, 1% (w/v) gelatin, 1 mM-MgCl₂, 0.2 mM of each dNTP, various amounts of purified DNA from rat hepatoma cDNA library, 2.5 units of AmpliTaq DNA Polymerase (Perkin–Elmer, Cetus Instruments) and $0.6 \mu\text{M}$ each of the primers GR8B [5'-GAT (T/C)TG ATG ATG ATG ATT AT-3'] and GR8C (5'-CCA

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione transferase; IPTG, isopropyl β -D-thiogalactoside; $1 \times$ SSPE, 0.15 M-NaCl/0.01 M-sodium phosphate (pH 7.4)/1 mM-EDTA; $1 \times$ SSC, 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0.

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The sequence for *Rattus* mRNA for glutathione transferase subunit 8 has been submitted to the EMBL Nucleotide Sequence Database under the accession number X62660.

GGC TGC AGG AAC TTC TT-3') (Operon Technologies Inc., Alameda, CA, U.S.A.). The reaction was started with 10 min at 95 °C and followed by 30 cycles of 1 min at 94 °C, 2 min at 54 °C and 3 min at 72 °C; 10 min at 72 °C ended the reaction.

Sequence analysis of PCR product

The 0.3 kb fragment obtained with PCR was recovered from a 2% (w/v) agarose gel after electrophoresis, purified with a Gene Clean Kit (BIO 101 Inc., La Jolla, CA, U.S.A.) and treated with T4 polynucleotide kinase. It was ligated blunt-ended in M13mp18 digested with *Sma*I and used to transform *Escherichia coli* XL-1 (Stratagene, La Jolla, CA, U.S.A.). Single-stranded DNA was prepared and a slightly modified protocol of Sanger's dideoxynucleotide method (Sanger *et al.*, 1977), involving Sequenase (U.S. Biochemical Corp., Cleveland, OH, U.S.A.), was used to determine the cDNA sequence.

Screening of λ gt10 rat hepatoma cDNA library

The PCR-derived fragment was labelled with [³²P]dCTP using Multiprime DNA labelling system (Amersham Corp.) according to the manufacturer's instructions. About 3×10^5 plaques were plated and replicas were made on nitrocellulose filters (Schleicher und Schuell, Dassel, Germany) essentially as described in Maniatis *et al.* (1982). Filters were hybridized at 55 °C for 16–20 h in 5 $\times$ SSPE (1 $\times$ SSPE is 0.15 M-NaCl, 0.01 M-sodium phosphate, pH 7.4, and 1 mM-EDTA), 5 $\times$ Denhardt's solution, 0.1% SDS and 0.1 mg sonicated single-stranded salmon sperm DNA/ml. Washes were carried out at room temperature and at 65 °C for 20 min in 0.1 $\times$ SSC and 0.1% SDS (1 $\times$ SSC is 0.15 M-NaCl and 0.015 M-sodium citrate, pH 7.0). Positive plaques were isolated, prepared according to standard procedures (Maniatis *et al.*, 1982) and purified with Lambda-Sorb (Promega).

Subcloning and sequence analysis

One clone, λ GTRA8, with an insert size of 0.9 kb was subcloned in pGEM-3Z (Promega) as well as in M13mp18 and M13mp19. DNA sequencing was performed as described above. By using an internal *Pst*I site as well as internal primers both strands were sequenced at least twice. Restriction sites were determined by standard methods and verified by computer analysis of the cDNA sequence.

Prokaryotic expression of rat GST 8-8

The entire cDNA sequence was amplified with PCR using a 5'-end-specific primer, GR8P (5'-TTC GAA TTC AGG ACA GGA GTC ATG GAA-3'), which was extended with an *Eco*RI linker and an oligo(dT) sequence extended with a *Sal*I linker for the 3'-end [5'-AAG TCG AC(T)₂₅-3']. Reaction conditions were as previously described. PCR yielded a fragment of expected size and the DNA was digested with *Eco*RI and *Sal*I, and subcloned into the prokaryotic expression vector pKK223-3 (Pharmacia LKB Biotechnology). pKK223-3 contains a bacterial ribosome binding site, and expression is driven by the *tac* promoter under control of the *lac* repressor. In addition, the primer GR8P contains two ribosome binding sites (AGGA).

Minipreparations of pKK223-3 containing PCR-derived cDNA, pKGTRA8, were prepared by standard methods (Maniatis *et al.*, 1982) and was restriction analysed. The cDNA was excised and subcloned in M13mp18 and M13mp19 for DNA sequencing as described above, to verify that no mutations had been introduced by PCR.

The expression construct was used to transform *E. coli* JM109. Transformants were picked and grown in 2 ml cultures in Luria

broth media (Maniatis *et al.*, 1982) with 50 μ g of ampicillin/ml. Induction with isopropyl β -D-thiogalactoside (IPTG), added to a final concentration of 1 mM, was carried out after 2 h of growth at 37 °C. Cultures were grown for an additional 5 h. Cells were harvested by centrifugation, suspended in 10 mM-Tris/HCl/1 mM-EDTA, pH 7.8, and sonicated for 20 s on ice using a Branson Sonifier model L equipped with a microtip at maximal power output (setting 7). Lysates were centrifuged for 10 min in a microcentrifuge, and portions of the supernatant were assayed for GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) as electrophilic substrate (Habig & Jakoby, 1981). Clones displaying GST activity were streaked out on Luria broth agar plates and used for 1-litre scale preparations of enzyme.

Enzyme purification

Cells from a 1-litre culture, transformed with pKGTRA8, induced with IPTG and grown overnight were harvested by centrifugation for 5 min at 5000 *g*. The bacterial pellet was washed with 50 mM-Tris/HCl, pH 7.8, containing 0.15 M-NaCl, and suspended in 10 mM-Tris/HCl, pH 7.8, with 1 mM-EDTA. Lysozyme (10 mg) was added, and the suspension was allowed to stand for 1 h at room temperature. The cells were sonicated (3 $\times$ 20 s) and centrifuged for 30 min at 25000 *g*. This procedure was repeated once. The combined supernatant fractions were desalted on a column (4 cm $\times$ 35 cm) of Sephadex G-25 (Pharmacia LKB Biotechnology Inc.) equilibrated with 10 mM-Tris/HCl, pH 7.8, containing 1 mM-EDTA and 1 mM-dithiothreitol (buffer A). Protein fractions were subjected to affinity chromatography on a GSH-Sepharose 6B gel prepared as described by Simons and Vander Jagt (1977). After being washed with buffer A fortified with 0.2 M-NaCl, enzyme was eluted with 5 mM-S-hexyl-GSH in buffer A. The affinity pool was dialysed overnight with several changes of buffer A. All purification steps were carried out at 4 °C.

Enzyme assays

Assay conditions were as described previously (Habig & Jakoby, 1981) with 1 mM-GSH and 1 mM-CDNB in 0.1 M-sodium phosphate buffer/1 mM-EDTA, pH 6.5. Peroxidase activities were measured in a coupled system containing 0.1 mM-NADPH, 0.6 unit of glutathione reductase, 1 mM-GSH and 1.5 mM-cumene hydroperoxide or 1.5 mM-H₂O₂ (Lawrence & Burk, 1976) at pH 6.5 or with 0.2 mM-linoleic acid hydroperoxide, in 0.1 M-sodium phosphate buffer, pH 7.0. Reactions were monitored spectrophotometrically at 340 nm. Activity with ethacrynic acid was measured with 0.25 mM-GSH and 0.2 mM-ethacrynic acid (Habig & Jakoby, 1981). Activity with 4-hydroxyalkenals was measured at 224 nm with 0.1 mM-aldehyde and 0.5 mM-GSH (Ålin *et al.*, 1985). Activities with acrolein and crotonaldehyde were measured at 216 and 226 nm respectively with 0.1 mM-aldehyde and 0.5 mM-GSH (Berhane & Mannervik, 1990; K. Berhane and B. Mannervik, unpublished work). Activities with 3-(adenin-9'-yl)propenal and 3-(uracil-1'-yl)propenal were measured at 262 and 295 nm respectively, with 0.1 mM-aldehyde and 1 mM-GSH (K. Berhane and B. Mannervik, unpublished work). Activity with 4-vinylpyridine was measured with 0.1 mM-alkene and 1 mM-GSH at 260 nm (K. Berhane and B. Mannervik, unpublished work). All assays with alkene substrates were carried out with 0.1 M-sodium phosphate, pH 6.5. Steroid isomerase activity was monitored at 248 nm in 25 mM-Tris/12.5 mM-K₂HPO₄, pH 8.5, with 0.068 mM-androstenedione, 0.1 mM-GSH and 0.1 mM-dithiothreitol (Benson *et al.*, 1977). CDNB, hydroperoxides, except H₂O₂, and ethacrynic acid were dissolved in ethanol, androstenedione and 3-(uracil-1'-yl)propenal were dissolved in methanol and 3-(adenin-9'-yl)propenal was dissolved in

The putative polyadenylation signal ATTTAA is between nucleotides 812 and 817. Identities between r8 and r1 are indicated by dashes, and exon boundaries in the subunit I gene (Telakowski-Hopkins *et al.*, 1986) are marked with vertical lines. Segments complementary to the PCR oligonucleotide primers GR8B and GR8C are underlined.

al., 1989), except for positions 18 and 48, which encode serine rather than valine and glycine rather than aspartate respectively. Re-evaluation of the results of the protein sequence analysis has revealed ambiguities (P. Ålin, personal communication) indicating that the primary structure presented in Fig. 1 is the correct one.

Expression of rat GST 8-8 in *E. coli*

The DNA encoding subunit 8 was subcloned for expression in the plasmid pKK223-3, yielding pKGTRA8, and the protein obtained was purified by affinity chromatography for structural and functional characterization.

A 1-litre culture of JM109 transformed with the expression vector pKK223-3 containing rat subunit 8 cDNA yielded approx. 2 mg of pure enzyme. SDS/PAGE demonstrated that the protein was homogeneous and that the apparent subunit M_r (24 500) was identical with that of GST 8-8 isolated from rat liver (results not shown).

The enzyme was further analysed by use of isoelectric focusing, and an isoelectric point of 6.8 was determined for the recombinant protein. This value is higher than that for the enzyme isolated from rat liver, which has been reported as 6.3. In rat tissues, the *N*-terminal methionine of subunit 8 is acetylated (Ålin *et al.*, 1989), but in the recombinant protein it was accessible to Edman degradation, and therefore unmodified. Thus, a gain of positive charge and consequently a higher isoelectric point should be expected in the recombinant protein. Determination of the *N*-terminal amino acid sequence gave the structure MEVK-PKLYYFQGRG, which is identical with that deduced from the nucleotide sequence.

A sample of the purified enzyme was subjected to hydrolysis and quantitative amino acid analysis. A specific absorption coefficient at 280 nm of $1 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ was determined for the protein on the basis of the amino acid composition and the absorption spectrum.

Catalytic properties

The activity of the recombinant rat GST 8-8 was tested with several commonly used substrates, and the specific activities were compared with previously published values (Table 1). In addition to the extraordinary activity towards 4-hydroxyalkenals reported earlier (Jensson *et al.*, 1986; Danielson *et al.*, 1987), the enzyme was also found to be highly efficient with other unsaturated aldehydes as substrates. GST 8-8 has an even higher activity with acrolein than that observed with human GST π , earlier reported as the most efficient isoenzyme in catalysing the conjugation between acrolein and GSH (Berhane & Mannervik, 1990). The acrolein (propenal) derivatives, crotonaldehyde, 3-(adenin-9'-yl)propenal and 3-(uracil-1'-yl)propenal, were also efficiently conjugated by the enzyme (Table 1).

Additional substrates active with other class Alpha GSTs (Mannervik *et al.*, 1985) were also tested. Cumene hydroperoxide, linoleic acid hydroperoxide and H_2O_2 were all found to give measurable activities with GST 8-8 (Table 1). The activity with H_2O_2 is noteworthy since this compound has been considered not to be a substrate for GSTs, as distinct from the selenium-dependent glutathione peroxidase (Mannervik, 1985a). Finally, a low, but significant, steroid isomerase activity was demonstrated with Δ^5 -androstene-3,17-dione.

Specificity constants

The kinetic constant k_{cat}/K_m was determined for a selected number of electrophilic substrates. This parameter has been determined previously for a series of 4-hydroxyalkenals with carbon chain lengths ranging from 5 to 15 atoms (Danielson *et*

Table 1. Specific activities of recombinant rat glutathione transferase 8-8 with some standard substrates and with compounds derivable by oxidative metabolism

Values are expressed as $\mu\text{mol}/\text{min}$ per mg of protein. For experimental details, see the text.

Substrate	Enzyme source ...	Specific activity		
		Recombinant {[GSH] in assay system (mM)}	Rat liver	
CDNB		10 (1.0)	10*	6† 15‡
Ethacrynic acid		9 (0.25)	7*	4.1† 9.5‡
4-Hydroxypentenol		50 (0.5)		
4-Hydroxynonenol		170 (0.5)	170*	150†
Acrolein		40 (0.5)		
Crotonaldehyde		17 (0.5)		
3-(Adenin-9'-yl)propenal		15 (1.0)		
3-(Uracil-1'-yl)propenal		10 (1.0)		
4-Vinylpyridine		0.2 (1.0)		
Cumene hydroperoxide		1 (1.0)	1.1*	
Linoleic acid hydroperoxide		0.08 (1.0)		
H_2O_2		0.25 (1.0)		
Δ^5 -Androstene-3,17-dione		0.06 (0.1)		

* Data taken from Ålin *et al.* (1989).

† Data taken from Adang *et al.* (1989).

‡ Data taken from Hayes (1986).

al., 1987). Here, α,β -unsaturated carbonyl compounds directly or indirectly related to the parent compound acrolein (propenal) were used as electrophilic substrates. The results are shown in Table 2 together with $\Delta\Delta G$ values calculated for the incremental transition state binding energy obtained by introducing substitutions in the acrolein molecule. The values were calculated from eqn. (1).

$$\Delta\Delta G = -RT \ln \frac{(k_{\text{cat}}/K_m)_{\text{RH}}}{(k_{\text{cat}}/K_m)_{\text{RX}}} \quad (1)$$

where RH represents acrolein (propenal) and RX the derivative (see Fersht, 1985). The experiments did not allow measurements with saturating concentrations of the second substrate, glutathione, because of the high non-enzymic reaction rate. Therefore the calculated parameters in Table 2 should be considered as semi-quantitative only.

DISCUSSION

Rat GST 8-8 was originally purified from tissues such as liver (Jensson *et al.*, 1986), lung (Robertson *et al.*, 1985) and kidney (Hayes, 1986), and it has subsequently been shown to be present in essentially all tissues investigated (Hayes & Mantle, 1986; Meyer *et al.*, 1989). The cDNA library from which the cDNA encoding GST 8-8 was isolated originated from hepatoma mRNA, indicating that the enzyme is also expressed in rat tumour cells. The enzyme appears to be more uniformly distributed among rat tissues than most other GSTs, suggesting that it serves a general cellular function. It has also been demonstrated that GST 8-8, like some of the other isoenzymes, is under hormonal control as indicated by the results of hypophysectomy in the rat (Mankowitz *et al.*, 1990). The availability of cDNA specific for this enzyme should facilitate studies of the control of the gene expression at the transcriptional and translational levels.

The cDNA clone, λGTRA8 , encoding GST 8-8 consists of 862 bp excluding a stretch corresponding to the poly(A) tract,

Table 2. Specificity constants for recombinant rat glutathione transferase 8-8 and calculated incremental binding energies in the transition state

The $k_{\text{cat.}}/K_m$ values were determined at 1 mM-GSH concentration. For experimental details, see the text. Values are means $\pm$ S.D. based on $n \geq 5$.

Substrate	$k_{\text{cat.}}/K_m$ (mm ⁻¹ ·s ⁻¹)	$\Delta\Delta G$ (kJ/mol)
Acrolein	208 $\pm$ 27	(0)
Crotonaldehyde	200 $\pm$ 20	-0.1
4-Hydroxypentenol	692 $\pm$ 9	3.0
4-Hydroxynonenol	3000 $\pm$ 80	6.7
3-(Uracil-1'-yl)propenal	92 $\pm$ 1	-2.0
3-(Adenin-9'-yl)propenal	138 $\pm$ 4	-1.0
Ethacrynic acid	325 $\pm$ 8	1.1

and appears to represent a full-length mRNA. The open reading frame of 666 bp corresponds to the amino acid sequence previously determined (Ålin *et al.*, 1989) except for residues 18 and 48 (see the Results section). The nucleotide sequence for these residues has been determined without ambiguity, and comparison with homologous class Alpha sequences (Mannervik & Danielson, 1988) support the assignments presented in Fig. 1.

GST 8-8 is the most divergent class Alpha isoenzyme, both in terms of primary structure and catalytic function (Ålin *et al.*, 1989). Amino acid sequence comparisons with rat subunits 1 (Y_a) and 2 (Y_b), human subunits B_1 (pGTH1 or λ GST2-3) and B_2 (pGTH2) as well as a mouse class Alpha subunit (Y_a) demonstrate positional identities in the range between 57 and 60% (Ålin *et al.*, 1989). Corresponding overall nucleotide comparisons demonstrate identities of 64–66% (Table 3). If the different sequences are divided into regions corresponding to the coding exons 2–7 of subunit 1 (Telakowski-Hopkins *et al.*, 1986), significant variation in the sequence similarities between the different exons are observed. Nucleotide sequences corresponding to exons 2 and 4 demonstrate the highest sequence identities, ranging between 71 and 78% (Table 3). The most divergent segment corresponds to exon 6 with nucleotide identities ranging between 57 and 61%. It is noteworthy that the highest identity value obtained (78%) was for rat subunit 8 and human subunit B_1 .

(pGTH1 or λ GST2-3) in the segment corresponding to exon 2. However, when compared over its full length, rat subunit 8 is approximately equally similar to other rat subunits of class Alpha as it is to the mouse and human subunits of this class. The 3'-non-coding regions of the sequences compared with the rat subunit 8 cDNA demonstrated only 24–32% sequence identity (Table 3). In summary, the analysis shows that rat subunit 8 is clearly homologous to other class Alpha subunits. The finding that it is equally closely related to rat as to human and mouse subunits suggests that it arose at a time in evolution preceding that for the origin of mammalian species.

Heterologous expression of GSTs has previously been achieved [see Widersten *et al.* (1991) and references cited therein]. Catalytically active proteins have been obtained and the enzymes are in most cases indistinguishable from those isolated from the natural sources. Also in the case of GST 8-8, the properties of the recombinant protein are indistinguishable from the enzyme isolated from rat liver, with the exception that the *N*-terminal amino acid of the latter is acetylated rather than unmodified (see Ålin *et al.*, 1989). This is probably the reason why the isoelectric point of the recombinant protein is at pH 6.8 rather than 6.3 reported previously (Ålin *et al.*, 1989). It is also noteworthy that the *N*-terminal amino acid of subunit 8 is the initiator methionine, whereas recombinant human class Alpha (Stenberg *et al.*, 1991) and class Mu (Widersten *et al.*, 1991) GSTs have been shown to lack this residue. This difference in *N*-terminal processing is consistent with the nature of the penultimate amino acid residue, as judged by comparison with other proteins, since small uncharged residues promote removal of the initiator methionine whereas bulky residues do not (Flinta *et al.*, 1986).

The availability of recombinant GST 8-8 will make more extensive functional studies possible. The corresponding cDNA can be used for construction of chimaeric enzymes with portions contributed by other enzymes. Such studies have already been made with other class Alpha GSTs (Widersten *et al.*, 1990). Chimaeric enzymes could be used for studies of structure-activity relationships and may give rise to proteins with novel catalytic properties. It has been proposed that exchange of exons corresponding to functional domains may have been a mechanism for generation of new catalytic activities in the evolution of GSTs (Mannervik, 1985b; Mannervik *et al.*, 1990).

GST 8-8 has special significance among the various GSTs,

Table 3. Nucleotide-sequence comparisons between cDNA clones for rat glutathione transferase 8-8 and other class Alpha glutathione transferases from rat, human and mouse tissues

Exons refer to coding sequences identified in the rat subunit 1 (Y_a) gene (Telakowski-Hopkins *et al.*, 1986).

Nucleotide structure	Sequence identity (%)					
	pGTB38*	pGTR261†	pGTB42‡	λ GST2-3§ or pGTH1	pGTH2	mY _a 1¶
Entire coding sequence	66	66	64	65	65	65
Exon 2	71	71	72	78	76	71
3	60	58	58	63	63	62
4	76	76	74	72	72	74
5	61	62	58	56	57	61
6	57	58	58	59	61	59
7	68	67	62	66	63	64
3'-Non-coding	32	32	33	29	24	25

* Rat subunit 1a (Y_a) (Pickett *et al.*, 1984).

† Rat subunit 1b (Y_b) (Lai *et al.*, 1984).

‡ Rat subunit 2 (Y_c) (Telakowski-Hopkins *et al.*, 1985).

§ Human subunit B_1 (Board & Webb, 1987; Tu & Qian, 1986).

|| Human subunit B_2 (H_a2) (Rhoads *et al.*, 1987).

¶ Mouse subunit 1 (Y_a) (Daniel *et al.*, 1987).

since it has activities with 4-hydroxyalkenals that are higher than almost every other substrate tested with any of the GSTs (Mannervik & Danielson, 1988). Further, 4-hydroxyalkenals are known products of oxidative metabolism (Benedetti *et al.*, 1980; Esterbauer *et al.*, 1982) and, in particular, lipid peroxidation, supporting the concept that GSTs in general are components of the cellular system providing protection against oxidative damage (Mannervik, 1985b, 1986, 1990). An enzyme corresponding to rat GST 8-8 has not been identified so far in other mammalian species, but the cDNA made available through the present work should make it possible to probe mRNA samples as well as cDNA and genomic libraries.

It has previously been demonstrated with a series of homologous 4-hydroxyalkenals that maximal catalytic efficiency is obtained with a carbon chain length of 11 or more atoms (Danielson *et al.*, 1987). These structure-activity studies have now been extended to other α,β -unsaturated carbonyl compounds (Table 1). It was found that all of the compounds tested serve as substrates and give specific activities that are similar or higher than that obtained with CDNB, the standard substrate which normally gives a specific activity higher by more than one order of magnitude than alternative substrates for the GSTs (Mannervik & Danielson, 1988). Among the substrates not previously tested, acrolein was found to give a high activity (Table 1). Acrolein has been found to be a particularly good substrate for human GST π (Berhane & Mannervik, 1990), but the specific activity for rat GST 8-8 (40 $\mu\text{mol}/\text{min}$ per mg) as compared with GST π (26 $\mu\text{mol}/\text{min}$ per mg) is somewhat higher. Base propenals, which may be generated from nucleic acids by radical reactions (Murugesan *et al.*, 1985), have previously been identified as substrates for human GSTs (Mannervik *et al.*, 1991; K. Berhane and B. Mannervik, unpublished work). The finding that these genotoxic compounds are efficiently conjugated by GST 8-8 extends the range of known substrates of pathophysiological significance for the enzyme.

In relation to its possible role in protection against toxic products of oxidative metabolism, it is also significant that model hydroperoxide substrates such as cumene hydroperoxide and linoleic acid hydroperoxide as well as H_2O_2 are substrates for GST 8-8 (Table 1). It is noteworthy that the enzyme displays activity with H_2O_2 , even if the activity is low in comparison with those of other active substrates, since activity with H_2O_2 in biological specimens has usually been ascribed exclusively to the selenium-dependent glutathione peroxidase (Mannervik, 1985a). Finally, it was found that the steroid isomerase activity was only a small fraction of that obtained with other class Alpha enzymes from the rat (Mannervik & Danielson, 1988).

The kinetic parameter k_{cat}/K_m is related to the binding affinity between the active site of the enzyme and the substrate in transition state (Fersht, 1985). Thus a more systematic study of compounds directly or indirectly related to the structure of acrolein was performed. It was found that addition of a methyl group to acrolein, giving rise to crotonaldehyde, did not significantly alter the binding affinity, whereas bulkier substituents such as those of the purine and pyrimidine rings of the base propenals effected small, but significant, decreases in binding affinity corresponding to -1.0 and -2.0 kJ/mol respectively. Addition of the hydroxyalkyl substituents of the 4-hydroxyalkenals afforded the most clearcut increase in binding affinity. In comparison with acrolein, the hydroxyhexyl substituent of 4-hydroxynonenal contributes 6.7 kJ/mol in increased binding affinity. It was also found that ethacrynic acid, which is a ketone rather than an aldehyde but which contains the structural element of acrolein, has a higher binding affinity in the transition state by 1.1 kJ/mol than has acrolein (Table 2). 4-Vinylpyridine has also been tested, but its specific activity was low in comparison with

the other alkene substrates (Table 1). The alkene group of 4-vinylpyridine is not conjugated with a carbonyl function, which may be an explanation for the lower activity.

In conclusion, the present studies of the cDNA for rat GST 8-8 reveal similarities as well as differences with respect to other class Alpha enzymes. The functional properties of the expressed protein, including the high activity with compounds that may be produced by oxidative metabolism, support the view that GST 8-8 has a prominent role in the cellular defence against oxidative insults in rat tissues.

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