Microsomal glutathione S-transferase A1-1 with glutathione peroxidase activity from sheep liver: molecular cloning, expression and characterization

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A 25 kDa subunit of glutathione S-transferase (GST) from sheep liver microsomes (microsomal GSTA1-1) with a significant selenium-independent glutathione peroxidase activity has been isolated and characterized. Several analytical criteria, including EDTA stripping, protease protection assay and extraction with alkaline Na_2CO_3, indicate that the microsomal GSTA1-1 is associated with the inner microsomal membrane. The specific cDNA nucleotide sequence reveals that the enzyme is made up of 222 amino acid residues and shares approx. 73–83 % sequence similarity to Alpha-class GSTs from different species. The molecular mass, as determined by electrospray mass ionization, is 25611.3 Da. The enzyme is distinct from the previously reported rat liver microsomal GST in both amino acid sequence and catalytic properties [Morgenstern, Guthenberg and DePierre (1982) Eur. J. Biochem. **128**, 243–248]. The microsomal GSTA1-1 differs from the sheep liver cytosolic GSTs, reported previously from this laboratory, in its substrate specificity profile and molecular mass [Reddy, Burgess, Gong, Massaro and Tu (1983) Arch. Biochem. Biophys. **224**, 87–101]. In addition to catalysing the conjugation of 4-hydroxynonenal with GSH, the enzyme also exhibits significant glutathione peroxidase activity towards physiologically relevant fatty acid hydroperoxides, such as linoleic and arachidonic acid hydroperoxides, as well as phosphatidylcholine hydroperoxide, but not with H_2O_2. Thus the microsomal GSTA1-1 isoenzyme might have an important role in the protection of biological membranes against oxidative damage.

Key words: Alpha-class GST, glutathione, 4-hydroxynonenal, lipid hydroperoxide, microsomes.

INTRODUCTION

The glutathione S-transferases (GSTs) (EC 2.5.1.18) are a complex family of multifunctional enzymes involved in the detoxification of a wide spectrum of compounds, such as environmental pollutants, carcinogens and mutagens, as well as endogenous toxic compounds such as lipid peroxidation products [1,2]. Most of the mammalian GSTs that have been purified and characterized are localized in the cytosol, where they exist as homodimers or heterodimers of subunits with molecular masses ranging from 24.5 to 28.5 kDa [3,4]. Subunits of GSTs are classified on the basis of a general nomenclature proposed by Mannervik et al. [5] and grouped into at least seven classes designated Alpha, Mu, Pi, Theta, Sigma, Zeta and Kappa on the basis of substrate and inhibitor specificities, antibody cross-reactivity and primary structures [1–9]. Whereas the cytosolic GSTs have been extensively investigated, there have been relatively few studies examining the occurrence, diversity and functional aspects of membrane GSTs, which have an ideal location to participate in the detoxification of lipid peroxidation products as well as lipophilic drugs and other xenobiotics.

In 1981 we isolated GSTs from rat liver microsomes that exhibited significant selenium-independent glutathione peroxidase (non-SeGPX) activity [10]. They are similar to the cytosolic GSTs in terms of subunit molecular mass, a finding consistent with those of other researchers, who merely reported their presence in the microsomes [11,12]. In 1982 a microsomal GST, distinct from cytosolic GSTs, was purified from rat liver microsomes by Morgenstern et al. [13]. This enzyme is different from the cytosolic GSTs in subunit molecular mass, its ability to undergo activation in the presence of thiol reagents [14] and its lack of primary sequence similarity [15] as well as immunological cross-reactivity [16,17]. Nevertheless, reports on the presence of GSTs resembling cytosolic isoenzymes in microsomal [18] and mitochondrial fractions [19] continue to attract attention, mainly owing to the potential role of the enzymes in the protection of membranes against oxidative damage.

As a part of our longstanding interest in the physiological role of non-SeGPX activity associated with the GSTs [20–22], we have now cloned and expressed a major cationic GST isoenzyme with non-SeGPX activity from sheep liver microsomes. We hypothesize that this membrane-associated cationic GST is a candidate for the protection of biological membranes against peroxidative damage. Sheep liver was chosen as the source for purifying microsomal GSTs because the GST-associated non-SeGPX activity represents approx. 98 % of the total GPX activity in sheep liver [23], which is similar to human liver in terms of the ratio of non-SeGPX to selenium-dependent glutathione per-

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; CHP, cumene hydroperoxide; CYP reductase, NADPH:cytochrome P450 reductase; GPX, glutathione peroxidase; GST, glutathione S-transferase; 4-HNE, 4-hydroxynonenal; NEM, *N*-ethylmaleimide; non-SeGPX, selenium-independent glutathione peroxidase; 13-*S*-HPODE, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadieonic acid.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession number AF140223.

oxidase activities [24]. This ratio is quite different from that in rat liver, where non-SeGPX represents only 10–15% of the total GPX activity [25].

EXPERIMENTAL

Materials

Δ^5-Androsterone-3,17-dione was synthesized as reported previously [23]. Polyclonal antibodies against the native sheep liver microsomal GSTA1-1 were prepared in rabbits at the Centralized Biological Laboratory (Pennsylvania State University, University Park, PA, U.S.A.), whereas the horseradish-peroxidase-linked mouse anti-(rabbit IgG), anti-calregulin and anti-(NADPH:cytochrome P450 reductase) [anti-(CYP reductase)] antibodies were purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.), Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and StressGen Biotechnologies (Vancouver, BC, Canada) respectively. *S*-HexylGSH was prepared and coupled to epoxy-activated Sepharose-6B as described by Reddy et al. [23]. The *S*-hexylGSH affinity-purified GSTs from rat and sheep liver cytosol were obtained as described previously [23]. 4-Hydroxynonenal (4-HNE) was purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Most of the chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Enzyme assays

The glutathione transferase assay was performed as described previously [26]. Bi-substrate kinetic experiments with 1-chloro-2,4-dinitrobenzene (CDNB) and GSH were performed by varying the CDNB concentration from 0 to 2 mM while keeping the GSH concentration constant at 2 mM. Enzyme activities are expressed as μmol of thioether formed per minute. Specificities towards other classical GST substrates were determined by the method of Habig and Jakoby [26]. For the comparison of specific activities of the microsomal GSTA1-1 with cytosolic cationic GSTs, enzyme assays with selected substrates were performed as described previously from our laboratory [23]. The microsomal GST conjugation activity with 4-HNE was determined in accordance with published procedures [27,28].

Glutathione peroxidase activities were measured in a coupled assay system containing 0.1 mM NADPH, 1 unit of GSH reductase, 1 mM GSH and 1.2 mM cumene hydroperoxide (CHP) or 0.25 mM H_2O_2 in 0.1 M potassium phosphate buffer, pH 7.0 [29]. In experiments where lipid hydroperoxides were used as substrates, CHP was replaced with the specific fatty acid hydroperoxide added in 50 μl of ethanol. Subcellular organelle marker enzymes, CYP reductase [30] and lactate dehydrogenase [31], were assayed as described previously. Protein concentrations were determined as described by Lowry et al. [32], with BSA as standard.

Synthesis of lipid hydroperoxides

13(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadecadieonic acid (13-*S*-HPODE) and 15(*S*)-hydroperoxy-5,8,11(*Z*),13(*E*)-eicosatetraenoic acid were prepared by incubating linoleic acid and arachidonic acid respectively with soybean lipoxygenase. 5(*S*)-Hydroperoxy-6(*E*),8,11,14(*Z*)-eicosatetraenoic acid and 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid were prepared by incubating arachidonic acid and linoleic acid respectively with potato 5-lipoxygenase as we described previously [33,34]. Phosphatidylcholine hydroperoxide was synthesized by incubating soybean phosphatidylcholine with soybean lipoxygenase, as reported previously [35].

Purification of GSTA1-1 from sheep liver microsomes

All protein purification steps were performed at 2–6 °C unless mentioned otherwise. A 10% (w/v) homogenate of a fresh sheep liver (100–200 g) was prepared in 25 mM Tris/HCl, pH 7.5, containing 0.25 M sucrose and 1 mM EDTA with a Waring blender. The resulting homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at 600 ***g*** for 20 min. The supernatant was subjected to centrifugation at 16000 ***g*** for 20 min and the resulting supernatant was centrifuged at 105000 ***g*** for 60 min. The microsomal pellet was resuspended by homogenization in 150 mM Tris/HCl, pH 7.5, with a Potter–Elvehjem homogenizer and centrifuged again at 105000 ***g*** for 60 min. This procedure was repeated three times to prepare thoroughly washed microsomes without any cytosolic contamination, which was assessed by the measurement of marker enzymes. The washed microsomal pellet was solubilized by the dropwise addition of Lubrol-Px to a final concentration of 4% under continuous stirring for 30 min; glycerol was then added to a final concentration of 20% (v/v). The sample was dialysed overnight against 100 vol. of 10 mM potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA and 5% (v/v) glycerol. The dialysate was centrifuged at 105000 ***g*** for 60 min to remove any insoluble residue. The supernatant was subjected to affinity chromatography on an *S*-hexylGSH-linked Sepharose-6B column as described by Reddy et al. [23]. The protein fractions exhibiting GST activity were concentrated, which is referred to as the affinity-purified GST mixture, and dialysed for 48 h against 600 vol. of 10 mM potassium phosphate buffer, pH 7.5, containing 5% (v/v) glycerol, with a minimum of three changes to remove the enzyme-bound *S*-hexylGSH completely. The microsomal GSTA1-1 was purified to homogeneity by CM-52 cation-exchange chromatography. The column-bound microsomal GSTA1-1 protein was eluted by a linear KCl gradient (0–0.2 M) and the fractions exhibiting both GST conjugation and non-SeGPX activities were pooled and concentrated. The native microsomal GSTA1-1 thus purified was used in all studies, including the preparation of polyclonal antibodies. The flow-through fractions, which had both 27 and 28 kDa GST subunits, were not characterized further because they had very low non-SeGPX activity.

Reverse-phase HPLC

Reverse-phase HPLC was performed on the CM-52-bound microsomal GSTA1-1 to check the identity and homogeneity of the sample as well as to compare the retention times with those of GSTs from sheep liver cytosol. A Supelcosil C_4 column (46 mm × 250 mm; pore size 30 nm) was used at a flow rate of 1 ml/min with a 35% (v/v) (solvent A) to 70% (v/v) (solvent B) gradient of acetonitrile containing 0.1% (v/v) trifluoroacetic acid over a period of 130 min; the eluate was monitored at 214 nm. The microsomal GSTA1-1 thus purified was used for amino acid sequence analyses of N-terminal, C-terminal and internal peptide fragments performed at the Mayo Clinic Protein Sequencing Facility (Rochester, MN, U.S.A.). The same GSTA1-1 was used for electrospray MS analyses performed at Pennsylvania State University's Intercollegiate Mass Spectrometry Center.

SDS/PAGE, isoelectric focusing and immunoblot analysis

SDS/PAGE was performed on slab gels [12.5% (w/v) gel] by Laemmli's method [36] at 100 V. Isoelectric focusing experiments were performed with precast gels (pI 3–10) in accordance with the instructions provided by the supplier (Invitrogen, Carlsbad, CA, U.S.A.). Immuno-blot analyses were performed by electrotransblotting the proteins from SDS/PAGE gels to nitrocellulose membranes by standard methods. The nitrocellulose membranes were incubated with the polyclonal anti-(sheep liver microsomal GSTA1-1) or anti-(17 kDa rat liver microsomal GST) antibodies at 1:9000 and 1:5000 dilutions respectively for 3 h at 25 °C. Anti-calregulin and anti-(CYP reductase) were used at 1:250 dilutions for 12 h at 4 °C. The horseradish-peroxidase-linked secondary anti-rabbit antibody was used at 1:2500 for 1 h at 25 °C and the membrane was developed with the enhanced chemiluminescence detection method (Pierce Chemical Co.).

Experimental criteria used for determining that GSTA1-1 isoenzyme is intrinsic to microsomes

Sepharose S-300 column-washing and stripping of microsomes with EDTA

To remove adsorbed ribosomes and other cytosolic proteins, including cytosolic GSTs from thoroughly washed sheep liver microsomes, the Sepharose S-300 column-washing procedure was adapted as described by Walter and Blobel [37]. After gel-filtration chromatography the microsomes were treated with 50 mM EDTA; the EDTA-stripped microsomes were collected by ultracentrifugation at 100000 ***g*** for 1 h [37]. The supernatant was precipitated with trichloroacetic acid for Western immunoblot analysis and 5 μg equivalents of the pellet and soluble fractions were used in each case. The membrane was probed with anti-(sheep microsomal GSTA1-1) as described in the previous section.

Protease protection assay

A triple-washed microsome suspension was subjected to trypsin digestion (30 μg of trypsin/mg of protein) in 0.2 ml of 50 mM potassium phosphate, pH 7.0, containing 20% (v/v) glycerol, 0.1 mM EDTA and 0.1 mM dithiothreitol at 25 °C for 30 min. The reaction was stopped by the addition of trypsin inhibitor (300 μg/mg of protein); the mixture was then centrifuged at 100000 ***g*** for 90 min at 4 °C. The pellet and supernatant fractions were used for Western immunoblot analysis as described above.

Extraction of membrane proteins with alkaline Na_2CO_3

The nature of membrane association of the microsomal GSTA1-1 was assessed by extraction, with alkaline Na_2CO_3 (0.1 M, pH 11.0), of freshly isolated and triple-washed sheep liver microsomes as described by other investigators for characterizing mitochondrial cytochrome P4501A1 [38,39]. The pellet and supernatant fractions were subjected to SDS/PAGE followed by Western immunoblot analyses as mentioned earlier.

Microsomal GSTA1-1 cDNA cloning

Approximately 25 mg of the frozen tissue was ground to a fine powder in a 1.5 ml Microfuge tube with a pestle under liquid nitrogen. The total RNA was extracted from the ground tissue by using the RNA isolation kit (Gentra, Minneapolis, MN, U.S.A.) and treated with DNase. First-strand cDNA was obtained from the total RNA preparation in a reverse transcriptase reaction with the SuperScript™ II RNase H^- reverse transcriptase from Gibco BRL (Life Technologies, Gaithersburg, MD, U.S.A.). All other reaction conditions were in accordance with the recommendation of the supplier. The cDNA obtained by the reverse transcriptase reaction was used as a template to amplify the coding sequence of the microsomal GSTA1-1. Gene-specific primers were synthesized on the basis of mammalian codon bias for the N-terminal and C-terminal amino acid sequences [40]. The forward primer for the PCR reaction was 5′-GTGGGCAAGCCCAAGCTGCACTACTTCAAC-3′, which corresponded to the first 10 residues of the N-terminal end of the enzyme. Similarly, the reverse primer specific for the last 11 residues of the C-terminal end was 5′-TTTAATCCTGAAAATCCTCTTTGCTTCTTCCAA-3′. *Pfu* turbo polymerase from Stratagene (La Jolla, CA, U.S.A.) was used wherever necessary. A single PCR product of approx. 670 bp was generated, which was cloned into a pGEM-T vector (Promega, Madison, WI, U.S.A.) and sequenced. The nucleotide sequence was subjected to a BLAST search and also translated to the corresponding amino acids with the DNA Strider version 1.0 software program of Dr C. Marck (Service de Biochemie et de Genteique Moleculaire, Gif-sur-Yvette, France).

Bacterial expression of recombinant microsomal GSTA1-1 from sheep liver

The open reading frame of the microsomal GSTA1-1 isoform was subcloned into a pET17b vector (Novagen) after appending *Nde*I and *Bam*HI sites into the cDNA. Bacterial expression host BL21(DE3)-competent cells (Novagen) were transformed with the pET17b-microsomal GSTA1-1 construct. Cells transformed with pET17b without insert were used as negative controls. One of the positive clones was grown in a 4-litre culture flask containing 1000 ml of Luria–Bertani broth and 100 μg/ml ampicillin at 37 °C, with continuous shaking at 150 rev./min. Protein expression, induced by the addition of 1 mM IPTG (isopropyl β-D-thiogalactoside) to the cells, was detected on an SDS/PAGE gel as well as by Western immunoblotting with the anti-(microsomal GSTA1-1) antibody. After 3 h of induction with IPTG, the cells were harvested by centrifugation at 6000 ***g*** for 10 min and washed in cold PBS. To 3.75 g (wet weight) of cells were added 25 ml of PBS containing 0.25 M sucrose, 5% (w/v) CHAPS and 0.5 ml of bacterial protease inhibitor cocktail (Sigma); the mixture was stirred at 4 °C for 3 h. The cell lysate was clarified by centrifugation at 10000 ***g*** for 30 min and the final volume was adjusted to 65 ml with cold PBS. The recombinant microsomal GSTA1-1 was purified by *S*-hexylGSH-linked Sepharose-6B affinity column chromatography as described previously. The kinetics of the GST and GPX activities were determined with the recombinant enzyme as described previously and compared with that of the purified native microsomal GSTA1-1.

RESULTS AND DISCUSSION

Localization and intrinsic nature of microsomal GSTA1-1

To verify the subcellular localization and to avoid contamination by cytosolic GSTs, microsomes were washed extensively while characteristic marker enzymes were monitored for each subcellular fraction. CYP reductase and lactate dehydrogenase activities were measured as marker enzymes for microsomes and cytosol respectively. As shown in Table 1, most of the CYP reductase activity was present in the microsomal fraction, whereas almost all the lactate dehydrogenase activity was recovered in the cytosolic fraction. The supernatant obtained from the final washing of the microsomal pellets contained very little GST and non-SeGPX activities. Lactate dehydrogenase activity could not be detected in the triple-washed microsomes, confirming a lack of cytosolic contamination.

To ascertain further that the microsomes were free of cytosolic GST and to verify the localization of microsomal GSTA1-1, we subjected microsomes to digestion with trypsin and washing on

Table 1 Activities of GST, GPX and subcellular marker enzymes during washing procedures on sheep liver microsomes

The cytosolic and microsomal fractions were prepared from 150 g (wet weight) of sheep liver. Enzyme activity values are representative of several experiments performed with different batches of sheep liver tissue. Microsomes were washed thoroughly three times and each time the contents were centrifuged at 105000 ***g*** for 60 min. The resulting supernatant at each washing was used for determining enzyme activities. The GST and GPX activities were assayed with CDNB and CHP as substrates respectively.

	Activity (μmol/min per g of tissue)			
Fraction	GST	GPX	LDH	CYP-reductase
16000 ***g*** supernatant	155.0	11.0	5.0	2.5
105000 ***g*** cytosol	151.0	10.4	4.9	0.1
First washings of microsomes	1.9	0.2	0.05	0.03
Second washings of microsomes	0.3	0.03	0.01	0.01
Third washings of microsomes	0.1	0.01	0	0
Microsomal pellet	1.0	0.3	0	2.3
Solubilized microsomes	2.2	0.4	0	4.2

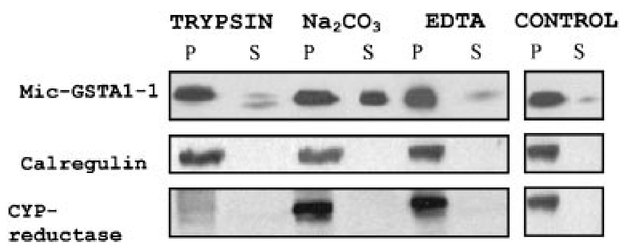

Figure 1 Microsomal localization and intrinsic nature of sheep liver microsomal GSTA1-1

The triple-washed microsomes were treated with trypsin, Na_2CO_3 and EDTA and then subjected to ultracentrifugation to obtain the pellet (P) and supernatant (S) fractions, which were analysed by SDS/PAGE and Western immunoblotting. Equal amounts of P and S fractions were loaded on SDS/PAGE gels; after transblotting, each of the blots was probed with anti-(microsomal GSTA1-1), anti-calregulin or anti-(CYP reductase) antibodies. As a control for the leaching of proteins out of the membrane, microsomes were resuspended in the respective buffers used in all treatments and centrifuged. The pellet and supernatant fractions were analysed for the presence of all the three proteins and are shown in the right panel in each case. Representative blot of $n = 3$.

a Sepharose S-300 column followed by EDTA treatment as described by Bhagwat et al. [39] and Walter and Blobel [37] respectively. The latter method has been successfully used to study the protein translocation into the closed vesicles of rough endoplasmic reticulum [37]. The EDTA treatment of microsomes from Sepharose S-300 column chromatography also showed that more than 90% of the GST protein was still retained in the microsomes (Figure 1). Limited proteolysis of intact microsomes by trypsin indicated that only a small amount of microsomal GSTA1-1 protein fragments was released into the supernatant, possibly owing to leaching of the protein from broken vesicles, whereas more than 90% of the microsomal GSTA1-1, resistant to digestion with trypsin, was found in the pellet fraction (Figure 1). The possible type of association of GSTA1-1 with the microsomal membrane was assessed by treatment with alkaline Na_2CO_3 (Figure 1), which has been shown to extract loosely bound membrane proteins while leaving strongly interacting proteins intact with the membranes [38]. The GSTA1-1 protein was partly extracted from the microsomes by treatment with Na_2CO_3, as the Western immunoblot results clearly showed that approx. 50% of the microsomal GSTA1-1

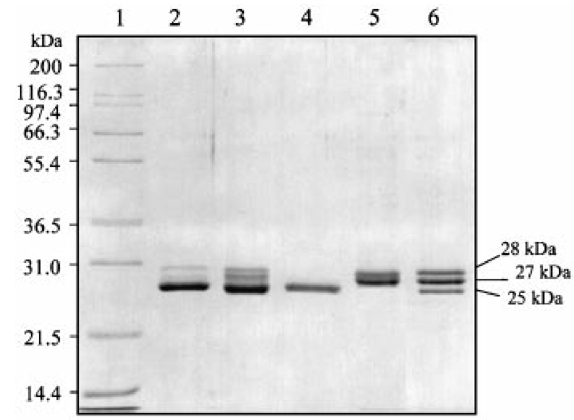

Figure 2 Comparison of the electrophoretic mobilities of *S*-hexylGSH affinity-purified GST isoenzymes from different sources by SDS/PAGE

Lane 1, molecular mass markers (molecular masses are indicated at the left); lane 2, sheep liver affinity-purified cytosolic GSTs; lane 3, sheep liver affinity-purified microsomal GSTs; lane 4, CM-52 bound fraction (cationic or microsomal GSTA1-1); lane 5, CM-52 flow-through fraction containing two major bands of anionic GSTs; lane 6, affinity-purified GSTs from rat liver cytosol.

protein still remained in the microsomes after treatment with Na_2CO_3 (Figure 1).

We tested the microsomal pellet and supernatant fractions of each of these treatments with antibodies against calregulin and CYP reductase, as controls. Calregulin is an integral protein located towards the lumen of the microsomal vesicle [41], whereas CYP reductase is also an integral protein, with a cytosolic orientation, anchored into the outer microsomal membrane only through its N-terminal transmembrane domain [42]. Results shown in Figure 1 clearly indicate that calregulin is not susceptible to tryptic cleavage owing to its internal nature, whereas CYP reductase, with its large cytoplasmic orientation, is completely cleaved by trypsin. However, the two enzymes were not extracted out of the membrane with either Na_2CO_3 or EDTA treatments, suggesting that they had an integral nature. These results clearly indicate that GSTA1-1 is located in the lumen of microsomes. Not only do these experiments prove the intrinsic nature of GSTA1-1 to microsomes but they also suggest a strong interaction of GSTA1-1 with microsomes (or microsomal proteins). However, the mechanism of integration of this GST isoenzyme in the microsomal membrane or its interaction with other microsomal proteins is as yet unknown and is under further investigation.

The triple-washed sheep liver microsomal preparation exhibited GST and non-SeGPX activities of 1.0 and 0.3 μmol/min per g of tissue respectively (Table 1). On solubilization with Lubrol-Px, the GST activity was doubled, whereas GPX activity was increased only marginally (Table 1). In addition, CYP reductase activity was enhanced 1.8-fold on solubilization. These results further support the contention that GSTA1-1 is integral to membranes.

Purification of microsomal GSTA1-1

The SDS/PAGE analysis of microsomal GST preparation obtained from *S*-hexylGSH-linked Sepharose-6B affinity chromatography displayed three protein bands corresponding to the microsomal GSTA1-1 (25 kDa) and the other two GST subunits of 27 and 28 kDa respectively (Figure 2, lane 3). This pattern is

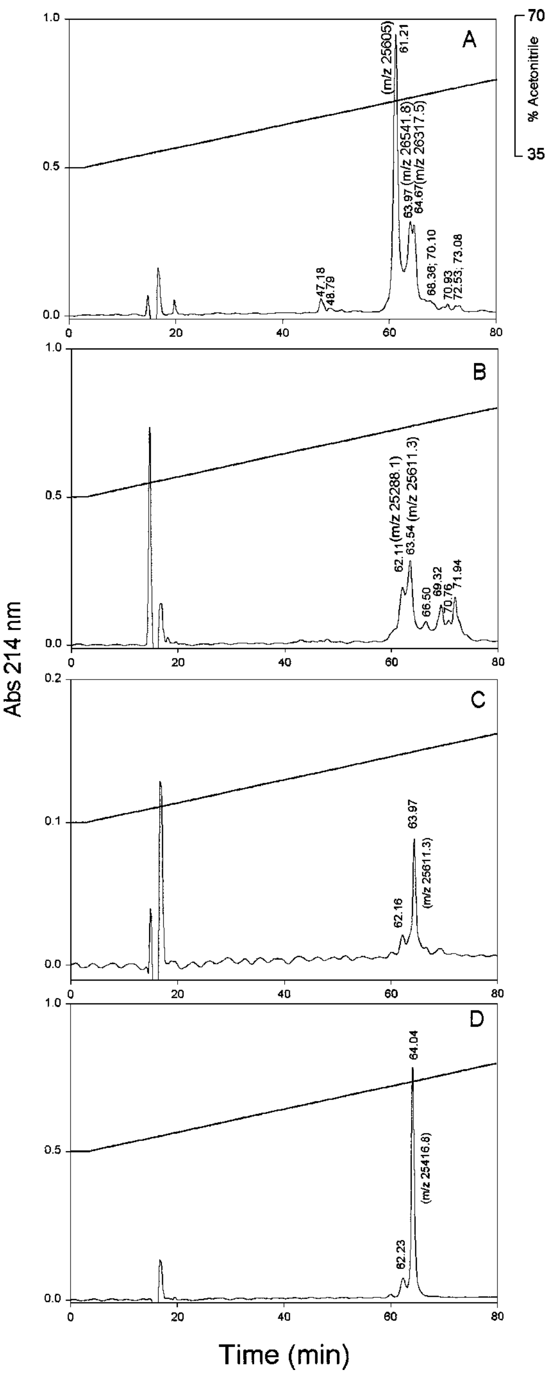

Figure 3 Reverse-phase HPLC of the *S*-hexylGSH affinity-column-purified sheep liver GSTs

Equal amounts of affinity-purified GSTs from cytosol and microsomes were applied to a C_4 column on the basis of A_{280} values: Cytosolic GST affinity mixture (**A**); microsomal GST affinity mixture (**B**); CM-52-purified native microsomal GSTA1-1 (**C**); CM-52-purified recombinant microsomal GSTA1-1 (**D**). The retention times and *m*/*z* values (in parentheses) of some of the major GST peaks are also indicated. This Figure shows representative chromatographs of three experiments. Abs214 nm, A_{214}.

similar to that found with the affinity-purified GSTs from sheep liver cytosol (compare lanes 2 and 3 in Figure 2) and rat liver cytosol (compare lanes 3 and 6 in Figure 2), although the relative distribution of GST subunits varied significantly between the three affinity-purified GST mixtures. The microsomal GSTA1-1 was purified to electrophoretic homogeneity by CM-52 ion-exchange chromatography (Figure 2, lane 4) and its purity was also verified by reverse-phase HPLC (see the Experimental section). The final yield of microsomal GSTA1-1 obtained from CM-52 column chromatography step was 30 % of the solubilized microsomal GST activity (results not shown).

1 GTG GGC AAG CCC AAG CTG CAC TAC TTC AAC GGA CGT GGC AGA ATG GAG TGC ATT CGG TGG 60
val gly lys pro lys leu his tyr phe asn gly arg gly arg met glu cys ile arg trp 20

CTC CTG GCT GCA GCC GGA GTG GAG TTT GAA GAG AAA TTT ATA GAA CAA CCA GAA GAC CTG 120
leu leu ala ala ala gly val glu phe glu glu lys phe ile glu gln pro glu asp leu 40

GAT AAG TTA AAA AAT GAT GGG AGT TTG ATG TTC CAG CAA GTG CCA ATG GTT GAA ATT GAT 180
asp lys leu lys asn asp gly ser leu met phe gln gln val pro met val glu ile asp 60

GGG ATA AAG CTG GTG CAG ACC AGA GCC ATT CTC AAC TAC GTT GCC ACT AAA TAT AAC CTC 240
gly ile lys leu val gln thr arg ala ile leu asn tyr val ala thr lys tyr asn leu 80

TAC GGG AAA GAC ATG AAG GAG AGA GCC CTG ATT GAT ATG TAC TCA GAG GGT GTG GCA GAT 300
tyr gly lys asp met lys glu arg ala leu ile asp met tyr ser glu gly val ala asp 100

TTG GGT GAA ATG ATC CTG CTT TTG CCA CTG TGC CCA CCT GAT CAA AAA GAT GCC AAA ATA 360
leu gly glu met ile leu leu leu pro leu cys pro pro asp gln lys asp ala lys ile 120

GCT CAG ATC AAA CAG AGT ACA ACA AAC CGT TAT CTC CCT GCA TTT GAA AAA GTG CTG AAG 420
ala gln ile lys gln ser thr thr asn arg tyr leu pro ala phe glu lys val leu lys 140

AGC CAC GGA CAA GAC TAT CTT GTG GGC AAC AAG CTG AGC AAG GCT GAC ATC CAC CTG GTT 480
ser his gly gln asp tyr leu val gly asn lys leu ser lys ala asp ile his leu val 160

GAA CTT CTC TAC CTT GTG GAA GAG CTG GAC CCT AGC CTT TTG GCC AAC TTC CCT CTG CTG 540
glu leu leu tyr leu val glu glu leu asp pro ser leu leu ala asn phe pro leu leu 180

AAG GGC CTG AAA GCC AGA GTC AGC AAT CTC CCG GCC GTG AAG AAG TTT CTG CAG CCT GGC 600
lys gly leu lys ala arg val ser asn leu pro ala val lys lys phe leu gln pro gly 200

AGC CAG AGG AAG CCT CCC ATG GAT GAG AAA ACT TTG GAA GAA GCA AAG AGG ATT TTC AGG 660
ser gln arg lys pro pro met asp glu lys thr leu glu glu ala lys arg ile phe arg 220

AT T AAA 666
ile lys 222

Figure 4 cDNA nucleotide sequence and deduced amino acid sequence of sheep liver microsomal GSTA1-1

The designed nucleotide sequences of the 5′ and 3′ ends corresponding to nt 1–30 and 639–666 respectively are based on the deduced amino acid information.

Reverse-phase HPLC analyses of *S*-hexylGSH affinity-purified GSTs from sheep liver cytosol and microsomes

The individual GST isoenzyme mixtures purified by affinity chromatography from sheep liver cytosol and microsomes were analysed by reverse-phase HPLC, and only the major peaks were subjected to electrospray ionization mass spectral analysis to determine their precise molecular mass (Figure 3). The overall separation patterns as well as the retention times of the cytosolic and microsomal GSTs were different. The chromatogram of the sheep liver microsomal affinity GST mixture revealed a protein peak at 63.54 min, which corresponded to GSTA1-1 with a molecular mass of 25611.3 Da (Figure 3B). This is in good agreement with the calculated mass of 25.2 kDa deduced from the specific cDNA nucleotide sequence. The chromatogram of sheep liver cytosolic affinity GST mixture revealed three protein peaks at 61.21, 63.97 and 64.67 min, which corresponded to 25605, 26541.8 and 26317.5 Da respectively (Figure 3A). The retention times for the purified microsomal GSTA1-1 and the recombinant microsomal GSTA1-1 were identical (Figures 3C and 3D). The molecular mass of the recombinant microsomal GSTA1-1 was found to be 25416.8 Da. The significant differences in mass values and the retention times between cytosolic GSTs and

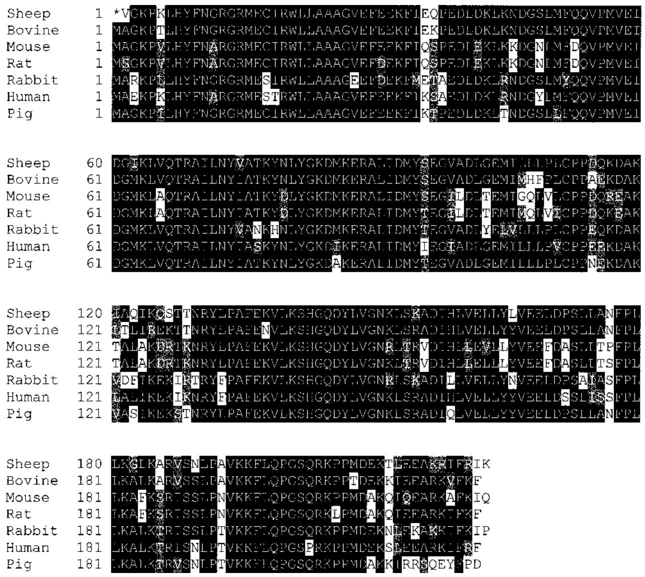

Figure 5 Amino acid sequence similarity of the sheep liver microsomal GSTA1-1 to other Alpha-class GSTs

The query string consisted of the complete amino acid sequences of sheep liver microsomal GSTA1-1 (GenBank® accession number AF140223), bovine corpus luteum GST subunit 2 (AF027386) and cytosolic Alpha-class GSTs from mouse (P 13745), rat ligandin chain 1 (P 00502), rabbit GST A1-1 (Q 08863), human GSTA1-1 (P 08263) and pig liver Alpha M14 GST (P 51781). Amino acid sequence identities are shown in black boxes; grey and white boxes represent conservative sequence differences and sequence dissimilarities respectively.

microsomal GSTA1-1 further support our contention that the microsomes were not contaminated by cytosolic GSTs.

Sequence analyses and cloning of microsomal GSTA1-1

The N-terminal and C-terminal amino acid sequences of the reverse-phase HPLC-purified microsomal GSTA1-1 were determined as Val-Gly-Lys-Pro-Lys-Leu-His-Tyr-Phe-Asn and Leu-Glu-Glu-Ala-Lys-Arg-Ile-Phe-Arg-Ile-Lys respectively. The sequence of an internal peptide was determined as Phe-Ile-Glu-Gln-Pro-Glu-Asp-Leu-Asp-Lys. Although the designed nucleotide sequence of the primers is based on the deduced amino acid information, the actual nucleotide sequence might vary to some extent. The complete amino acid sequence of the enzyme was deduced from the specific cDNA nucleotide sequence of 666 bp microsomal GSTA1-1 (Figure 4). The sequence of the internal peptide was found at position 33 from the N-terminal end of the deduced sequence. The protein contained 222 residues and had a calculated molecular mass of 25.2 kDa. The deduced sequence

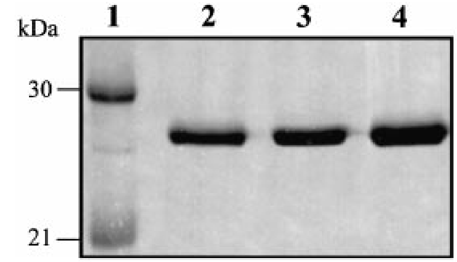

Figure 6 SDS/PAGE of purified recombinant microsomal GSTA1-1

Lane 1, molecular mass markers (molecular masses are indicated at the left); lane 2, sheep liver native microsomal GSTA1-1; lane 3, recombinant microsomal GSTA1-1 purified by affinity chromatography; lane 4, recombinant and native microsomal GSTA1-1 mixed together. Representative gel of $n = 4$.

showed that Leu and Lys were predominant amino acid residues, with molar percentages of 14.9 and 10.8 respectively. In addition, the amino acid sequence also indicates that 35–40 mol % of the residues were hydrophobic. Comparison of the N-terminal sequence with other GSTs revealed a very close match in the amino acids Gly-Lys-Pro-Lys-Leu-His-Tyr-Phe-Asn starting from position 2 (Figure 5). A BLAST search of the complete amino acid sequence showed 73–83 % similarity to various class Alpha GSTs from different species (Figure 5). The C-terminal sequence of the microsomal GSTA1-1 matches closely with bovine corpus luteum GST (Figure 5). Furthermore, the amino acid residues Met-208, Leu-213, Ala-216 and Phe-220, which line the hydrophobic electrophile binding in hGSTA1-1 [43], are also conserved in the microsomal GSTA1-1 (Figure 5). A comparison of the GSH-binding site, which constitutes approximately the first 100 residues of the N-terminus in the microsomal GSTA1-1, with different GST classes also indicates that the amino acid residues that interact with different functional groups of GSH substrate are conserved [1]. Of significance is the tyrosine residue that occurs at position 8 in the microsomal GSTA1-1: it has been shown to be involved in enhancing the nucleophilicity of the thiol group of GSH in other Alpha-class GSTs [44].

Bacterial overexpression of microsomal GSTA1-1

The recombinant microsomal GSTA1-1 enzyme was purified from BL21 (DE3) cell lysate in its active form by using the *S*-hexylGSH affinity column to electrophoretic homogeneity, with a final yield of 96 %. We obtained 67 mg of pure recombinant microsomal GSTA1-1 per litre of culture medium (Table 2). The specific activities were determined as 26 μmol/min per mg and 3.6 μmol/min per mg with CDNB (conjugation) and CHP

Table 2 Purification of recombinant sheep liver microsomal GSTA1-1 expressed in BL21(DE3)

The GST and GPX activities were assayed with CDNB and CHP as substrates respectively. These values are representative of several protein expression experiments.

		Total enzyme activity (μmol/min)		Total specific activity (μmol/min per mg)			
Fraction	Total protein (mg)	GST	GPX	GST	GPX	Fold purification	Yield (%)
Crude cell lysate	345	1806	293	5.2	0.8	1.0	100
Lysate after centrifugation	179	1755	373	9.8	2.1	1.9	97
Affinity pool	78	1739	239	22.6	3.1	4.3	96
Affinity pool after dialysis	67	1739	242	26.0	3.6	5.0	96

Table 3 Comparison of substrate specificities of the microsomal GSTA1-1 with those of other cationic sheep liver cytosolic GSTs

The specific activities of cationic GSTs (C-1 to C-7) from sheep liver cytosol were previously reported [23]. Enzyme activities with C-4 and the microsomal GSTA1-1 are shown in bold. Results are means ± S.D. for triplicate assays. Abbreviations: DCNB, 1,2-dichloro-4-nitrobenzene; 9-*S*-HPODE, 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; MicGST, native microsomal GSTA1-1.

	Enzyme activity (μmol/min per mg of protein)							
Substrate	C-1	C-2	C-3	**C-4**	C-5	C-6	C-7	**MicGST**
CDNB	8.9	9.7	14.1	**26.9**	7.6	9.3	6.5	**25.8±1.8**
DCNB	0.19	0.11	0.12	**0.10**	0.14	0.09	0.05	**0.15±0.05**
Bromosulphothalein	0.12	0.14	0.10	**0.08**	0.31	1.48	0.81	**0.28±0.06**
p-Nitrobenzyl chloride	0.0	0.15	0.24	**0.50**	0.0	0.0	0.15	**0.61±0.18**
Δ^5-Androsterone-3,17-dione	0.09	0.13	0.13	**0.13**	0.08	0.04	0.55	**1.0±0.1**
Ethacrynic acid	0.45	0.62	0.25	**0.17**	0.48	0.37	0.50	**0.11±0.05**
p-Nitrophenyl acetate	0.13	0.04	0.08	**0.07**	0.18	0.13	0.24	**0.16±0.09**
9-*S*-HPODE	6.2	2.7	4.1	**6.3**	1.5	2.9	3.6	**0.5±0.08**
CHP	8.3	4.5	4.6	**9.2**	11.3	7.5	6.1	**4.2±0.5**

(peroxidase) as substrates respectively (Table 2). The relative mobilities of the purified recombinant microsomal GSTA1-1 and the native microsomal GSTA1-1 on an SDS/PAGE gel were identical, with a subunit molecular mass of 25.2 kDa (Figure 6). In addition, both native and recombinant microsomal GSTA1-1 had almost identical molecular masses as determined by electrospray ionization MS and by their retention times on the reverse-phase HPLC (Figures 3C and 3D). The recombinant microsomal GSTA1-1 existed as homodimers in solution as determined by dynamic light-scattering experiments (results not shown). To confirm that the native and recombinant enzymes were identical, the isoelectric points, substrate specificities and kinetic parameters of the conjugation of GSH with CDNB and the reduction of hydroperoxides (CHP and 13-*S*-HPODE) were compared.

Characterization of native microsomal GSTA1-1 from sheep liver microsomes and its comparison with the recombinant microsomal GSTA1-1

Substrate specificity and kinetic parameters

The substrate specificity of the native microsomal GSTA1-1 was compared with that of seven cationic cytosolic GSTs (Table 3) previously reported from our laboratory [23]. The microsomal GSTA1-1 resembled the cytosolic C-4 isoenzyme in terms of its enzyme activities with CDNB, 1,2-dichloro-4-nitrobenzene, *p*-nitrobenzyl chloride and ethacrynic acid; however, the activities towards such other substrates as bromosulphothalein, *p*-nitrophenyl acetate Δ^5-androsterone-3,17-dione, 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid and CHP differ from the cytosolic C-4 isoenzyme (Table 3). These results indicate clearly that the microsomal GSTA1-1 characterized in these studies is different from the GSTs previously characterized from sheep liver cytosol.

Kinetic parameters for CDNB by native and recombinant microsomal GSTA1-1 enzymes were determined with a view to showing that the two enzymes were kinetically identical. The CDNB conjugation reaction followed hyperbolic increases in enzyme activity with increased substrate concentration. The K_m and V_{max} values for the native microsomal GSTA1-1 with CDNB were 48 μM and 31 μmol/min per mg of protein respectively and the values for the recombinant microsomal GSTA1-1 were 38 μM and 35 μmol/min per mg of protein respectively. The substrate kinetics with CHP and 13-*S*-HPODE were also measured to ascertain the identity of the two enzymes with respect to the GPX activity. The K_m and V_{max} with CHP for the native enzyme were calculated as 87.9 μM and 7.5 μmol/min per mg respectively and the values for recombinant microsomal GSTA1-1 were very similar at 83 μM and 7.2 μmol/min per mg respectively. The K_m/V_{max} ratio with 13-*S*-HPODE, a physiologically relevant substrate, for the native and recombinant enzymes was 5.2 and 6.5 respectively. In addition, specific activities of the two enzymes were compared with a wide range of conventional GST and GPX substrates of physiological relevance. As shown in Table 4, the two enzymes exhibit identical catalytic activities in terms of both conjugation (GST) and peroxidase (GPX) reactions. Most importantly, significant and comparable values of catalytic activities towards 4-HNE conjugation and lipid hydroperoxide reduction were observed with both native and recombinant enzymes (Table 4). In addition, neither enzyme used H_2O_2 as a substrate in the GPX reaction (Table 4). Similarly, *N*-acetylcysteine could not displace GSH as a substrate in either the conjugation or the peroxidase reaction. The isoelectric point of native microsomal GSTA1-1 was determined as 9.2, whereas that of the recombinant microsomal GSTA1-1 was 9.3. These studies unequivocally

Table 4 Comparison of the substrate specificity of the native sheep liver microsomal GSTA1-1 with that of recombinant microsomal GSTA1-1

The hydroperoxide concentrations were as follows: CHP, 1.2 mM; the indicated lipid hydroperoxides, 0.1 mM; H_2O_2, 0.25 mM. *N*-Acetylcysteine was substituted for GSH at a concentration of 10 mM. The reaction was initiated by the addition of 1 μg of pure enzyme. In experiments in which phosphatidylcholine hydroperoxide (PtdCho-OOH) was used, an equimolar concentration of SDS was added to disperse the substrate uniformly. Results are means ± S.D. for triplicate assays. Abbreviations: 5-*S*-HPETE, 5(*S*)-hydroperoxy-6(*E*),8,11,14(*Z*)-eicosatetraenoic acid; 15-*S*-HPETE, 15(*S*)-hydroperoxy-5,8,11(*Z*),13(*E*)-eicosatetraenoic acid; 9-*S*-HPODE, 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 13-*S*-HPODE, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadieonic acid; Rec-GST, recombinant microsomal GSTA1-1.

	Specific activity (μmol/min per mg of protein)	
Substrate	Native microsomal GSTA1-1	Rec-GST
CDNB	25.8 ± 1.8	25.2 ± 1.4
4-HNE	1.9 ± 0.06	2.2 ± 0.13
N-Acetylcysteine	0.0	0.0
CHP	4.2 ± 0.5	4.2 ± 1.2
9-*S*-HPODE	0.5 ± 0.08	0.4 ± 0.05
13-*S*-HPODE	2.2 ± 0.017	2.5 ± 0.15
5-*S*-HPETE	0.6 ± 0.015	0.5 ± 0.025
15-*S*-HPETE	0.8 ± 0.046	0.8 ± 0.03
PtdCho-OOH	0.6 ± 0.25	0.6 ± 0.3
H_2O_2	0.0	0.0

Table 5 Kinetic parameters of the recombinant microsomal GSTA1-1

K_m and V_{max} values were obtained from Lineweaver–Burk plots by linear regression analysis. The GST conjugation activity with 4-HNE was performed by varying the reactive aldehyde concentration from 0.5 to 100 μM while the GSH concentration was kept constant at 0.5 mM. The Non-SeGPX (reduction) activity was measured with a non-physiological substrate, CHP, as well as with the physiologically relevant substrates 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadieonic acid (13-*S*-HPODE), 5(*S*)-hydroperoxy-6(*E*),8,11,14(*Z*)-eicosatetraenoic acid (5-*S*-HPETE), 15(*S*)-hydroperoxy-5,8,11(*Z*),13(*E*)-eicosatetraenoic acid (15-*S*-HPETE) and phosphatidylcholine hydroperoxide (PtdCho-OOH) as mentioned in the Experimental section. The amount of GST protein in each assay was 1.0 μg in a total reaction volume of 1.2 ml. Results are means ± S.D. for triplicate assays.

Substrate	K_m (μM)	V_{max} (μmol/min per mg)
GSH	62.0 ± 15	34.0 ± 3
CDNB	38.0 ± 7	35.0 ± 2
4-HNE	25.0 ± 7.2	3.7 ± 0.45
CHP	83.0 ± 10	7.2 ± 0.5
5-*S*-HPETE	11.5 ± 4.9	1.1 ± 0.4
13-*S*-HPODE	18.0 ± 1.2	3.5 ± 0.8
15-*S*-HPETE	27.6 ± 7.7	1.8 ± 0.8
PtdCho-OOH	8.5 ± 2.9	0.8 ± 0.2

confirm that the recombinant enzyme is identical with the native microsomal GSTA1-1. In all further studies we therefore used the recombinant microsomal GSTA1-1 to characterize the enzyme in terms of the peroxidase activity towards various fatty acid hydroperoxides, including phosphatidylcholine hydroperoxide, and the effect of thiol activators such as *N*-ethylmaleimide (NEM) and GSSG.

Kinetic parameters of the recombinant sheep liver microsomal GSTA1-1

The K_m values for the physiologically relevant lipid hydroperoxides were calculated to be lower than those determined for CHP, a non-physiological substrate, which suggests that the microsomal GSTA1-1 might have a significant detoxification role in protecting membranes from lipid peroxidation and the resulting reactive secondary products. Comparison of the kinetic parameters with each of these lipid hydroperoxide substrates indicates that the V_{max} with 13-*S*-HPODE is the highest, whereas the K_m with 15(*S*)-hydroperoxy-5,8,11(*Z*),13(*E*)-eicosatetraenoic acid is higher than with other lipid hydroperoxides (Table 5). Another significant catalytic property of this enzyme is the peroxidase activity towards phosphatidylcholine hydroperoxide, a phospholipid hydroperoxide substrate commonly found in cellular membranes during oxidative stress. The K_m and V_{max} for phosphatidylcholine hydroperoxide were calculated to be 8.5 μM and 0.8 μmol/min per mg of protein respectively (Table 5), suggesting that the enzyme can readily reduce membrane-bound phospholipid hydroperoxides. It is therefore conceivable that the microsomal GSTA1-1 protects the membranes from the inner side, whereas the 17 kDa microsomal GST, which faces the cytosol, provides protection against oxidant species from the outside. In addition, the specific activities towards some of the physiologically relevant lipid hydroperoxides are much higher for the microsomal GSTA1-1 than those reported for the rat and human liver microsomal 17 kDa GST [45,46]. Nevertheless, the non-SeGPX activity associated with the microsomal GSTA1-1 and 17 kDa GSTs might offer a synergistic protection against membrane oxidative damage in cells. The conjugation of 4-HNE with GSH by the recombinant GSTA1-1 is another physiologically important catalytic property of this enzyme, although its activity is not as high as in the cytosolic GSTA4-4 enzyme [47]. In comparison with the microsomal 17 kDa GST in rat, the sheep liver microsomal GSTA1-1 shows 3-fold higher activity towards 4-HNE [48], whereas the human and mouse 17 kDa GSTs completely lack conjugation activity towards 4-HNE [18,48]. The significant conjugation activity towards 4-HNE, a cytotoxic and mutagenic lipid peroxidation product of oxidative stress that is linked to the pathophysiology of many degenerative diseases, including aging, atherosclerosis, Parkinson's disease and Alzheimer's disease [47], might therefore be an additional antioxidant property of the GSTA1-1 localized in the microsomes.

Effect of NEM, GSSG and *S*-alkylGSH

Incubation of the recombinant microsomal GSTA1-1 with NEM (0.05–1.0 mM) did not have any effect on the CDNB conjugation activity (Table 6), whereas the treatment with GSSG caused an approx. 25 % decrease in the enzyme activity at concentrations between 2.5 and 10.0 mM (Table 6). The effects of NEM and GSSG on the native sheep liver microsomal GSTA1-1 activity were similar to those observed with the recombinant enzyme (results not shown). These results are in contrast with the microsomal 17 kDa GST, which is activated severalfold by NEM and GSSG [14,16,17,49]. This clearly indicates that the covalent modification of either one or both cysteine residues does not bring about significant conformational change in the microsomal GSTA1-1, which is thought to be responsible for the activation of 17 kDa GST. In addition, we have investigated the effect of *S*-alkylGSH derivatives, with alkyl side-chain lengths ranging from C_2 (ethyl) to C_{10} (decyl), on the conjugation activity of recombinant microsomal GSTA1-1. The IC_{50} values for inhibition by each of these compounds indicate that *S*-decylGSH was the most

Table 6 Effect of NEM and GSSG on the conjugation activity of recombinant microsomal GSTA1-1

The test compound was incubated with the recombinant microsomal GSTA1-1 enzyme at the indicated concentration for 1 min before the standard GST assays were performed with CDNB as a substrate. Results are means ± S.E.M. for five replicates. *Significantly different from control value ($P < 0.05$).

Compound	Concentration (mM)	Specific activity (μmol/min per mg of protein)	Change (% of control)
NEM	0.0	19.9 ± 0.42	0
	0.05	19.4 ± 0.49	−2.5
	0.25	20.2 ± 0.40	+1.5
	1.0	19.5 ± 0.81	−2.1
GSSG	0.0	21.8 ± 0.77	0
	0.5	21.7 ± 0.14	−0.4
	2.5	20.4 ± 0.38	−6.6
	5.0	20.0 ± 0.32	−8.5
	10.0	18.3 ± 0.34*	−16.2

Table 7 Effect of chain length of *S*-alkylGSH on inhibition of conjugation activity of recombinant microsomal GSTA1-1

Results are representative of four experiments.

Compound	Inhibitor concentration range (μM)	IC_{50} (μM)
S-EthylGSH	0–5000	3800.0
S-HexylGSH	0–50	1.5
S-DecylGSH	0–10	0.3

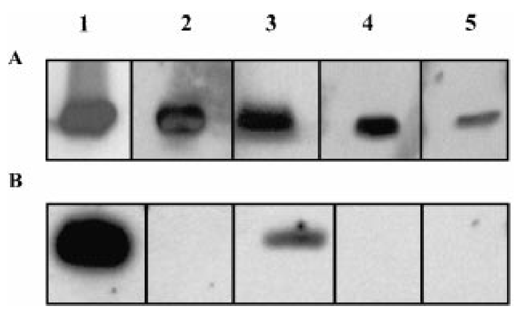

Figure 7 Immunological cross-reactivity between GSTs from different sources

Lane 1, rat liver solubilized microsomes; lane 2, sheep liver cytosolic affinity GST mix; lane 3, sheep liver solubilized microsomes; lane 4, purified native sheep liver microsomal GSTA1-1; lane 5, recombinant sheep liver microsomal GSTA1-1. Identical blots were probed with polyclonal antibodies prepared against sheep liver microsomal GSTA1-1 (**A**) and rat liver microsomal GST (17 kDa) (**B**). Conditions for the immunoblot were as described in the Experimental section. Representative blot of $n = 3$.

potent inhibitor, with an IC_{50} value of 0.3 μM; decreasing inhibitory effects were observed with decreasing chain length of the alkyl group. *S*-EthylGSH inhibited the microsomal GSTA1-1 the least, with an IC_{50} of 3.8 mM (Table 7). These semi-quantitative structure–activity relationship studies with *S*-alkylGSH compounds indicate that *S*-decylGSH inhibits the enzyme more strongly than the short-chain-length alkylGSH derivatives, suggesting that the substrate-binding site is hydrophobic. These studies are further confirmation of why microsomal GSTA1-1 exhibits significant enzyme activities towards a wide spectrum of lipid peroxidation products, including 4-HNE and lipid hydroperoxides. In addition, it is noteworthy that *S*-hexylGSH, which is used as a ligand in the affinity chromatography column for the purification of GSTs from solubilized microsomes, does not bind 17 kDa GST, probably owing to differences in the architectures of these two GSTs with regard to their substrate-binding (G and H) sites.

Immunoblot analysis

Immunoblot analyses with the antisera prepared against native sheep liver microsomal GSTA1-1 and rat liver microsomal 17 kDa GST were performed to determine the immunological cross-reactivity of the microsomal GSTA1-1 with other GSTs from cytosol. Figure 7 shows that the anti-(sheep liver microsomal GSTA1-1) antibodies cross-reacted with the rat liver microsomal GSTA1-1 (Figure 7A, lane 1) as well as with the GST present in sheep liver cytosolic affinity-purified GST mixture (Figure 7A, lane 2). Western immunoblot analysis with the anti-(17 kDa GST) antibodies detected the 17 kDa microsomal GST in solubilized rat and sheep liver microsomes (Figure 7B, lanes 1 and 3) and also in the flow-through fractions of the *S*-hexylGSH affinity column (results not shown). However, the antibodies prepared against 17 kDa GST did not cross-react with the native or recombinant microsomal GSTA1-1 proteins (Figure 7B, lanes 4 and 5). Comparison of the sheep liver microsomal GSTA1-1 with Alpha-class GSTs from other mammalian sources such as mouse, rat, rabbit, human liver and bovine corpus luteum show amino acid sequence similarity ranging from 73–83 % (Figure 5). This explains the immunological cross-reactivity of anti-(microsomal GSTA1-1) polyclonal antibodies with the cytosolic GST proteins but not with the 17 kDa GST, because there is no sequence homology between the two microsomal GST isoenzymes. The presence of large stretches of conserved amino acid residues, such as domains that form the substrate-binding site, among various GSTs including the microsomal GSTA1-1 also suggests a common evolutionary link.

Western immunoblot analyses of microsomes prepared from RAW264.7 murine macrophages, U937 human macrophages and HepG2 human hepatoblastoma cells, in addition to whole tissue sources such as canine pancreas and human liver, indicated that the microsomal GSTA1-1 is present in all cell types tested (results not shown).

Conclusions

In summary, we have reported here a microsomal GST from sheep liver, designated microsomal GSTA1-1, that differs from the 17 kDa rat and human liver microsomal GSTs with regard to molecular size, catalytic properties, immunological cross-reactivity and its inability to be activated by thiol-modifying reagents. Although the microsomal GSTA1-1 isoenzyme is similar to cytosolic Alpha-class GSTs with regard to the sequence and immunological cross-reactivity, the microsomal localization and its broad substrate specificity make this isoenzyme a unique member of Alpha-class GSTs. The significant non-SeGPX activity towards lipid hydroperoxides and GST conjugation activity with 4-HNE makes the microsomal GSTA1-1 isoenzyme physiologically very important.

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