Mammalian class Sigma glutathione S-transferases: catalytic properties and tissue-specific expression of human and rat GSH-dependent prostaglandin D\(_2\) synthases

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GSH-dependent prostaglandin D\(_2\) synthase (PGDS) enzymes represent the only vertebrate members of class Sigma glutathione S-transferases (GSTs) identified to date. Complementary DNA clones encoding the orthologous human and rat GSH-dependent PGDS (hPGDS and rPGDS, respectively) have been expressed in *Escherichia coli*, and the recombinant proteins isolated by affinity chromatography. The purified enzymes were both shown to catalyse specifically the isomerization of prostaglandin (PG) H\(_2\) to PGD\(_2\). Each transferase also exhibited GSH-conjugating and GSH-peroxidase activities. The ability of hPGDS to catalyse the conjugation of aryl halides and isothiocyanates with GSH was found to be less than that of the rat enzyme. Whilst there is no difference between the enzymes with respect to their \(K_m\) values for 1-chloro-2,4-dinitrobenzene, marked differences were found to exist with respect to their \(K_m\) for GSH (8 mM versus 0.3 mM for hPGDS and rPGDS, respectively). Using molecular modelling techniques, amino acid substitutions have been identified in the N-terminal domain of these enzymes that lie outside the proposed GSH-binding site, which may explain these catalytic differences. The tissue-specific expression of PGDS also varies significantly between human and rat; amongst the tissues examined, variation in expression between the two species was most apparent in spleen and bone marrow. Differences in catalytic properties and tissue-specific expression of hPGDS and rPGDS appears to reflect distinct physiological roles for class Sigma GST between species. The evolution of divergent functions for the hPGDS and rPGDS is discussed in the context of the orthologous enzyme from chicken.

Key words: detoxification, gene expression, prostaglandin synthesis.

INTRODUCTION

Prostaglandins (PGs) are a widely distributed group of oxygenated eicosanoids that modulate cellular function in both a physiological and pathological context [1]. The biosynthesis of PGs occurs through multiple enzymatically-regulated reactions. The process is initiated through the release of arachidonic acid (AA) from membrane phospholipids, a reaction catalysed by phospholipases. Subsequently, AA is converted to an unstable PGH\(_2\) intermediate by the actions of PG endoperoxide synthase, also called cyclooxygenase (COX). Two distinct COX isoenzymes exist, COX-1 and COX-2, which are differentially regulated [2]. Once formed by COX-1 or COX-2, the PGH\(_2\) intermediate may be converted into either thromboxane A\(_2\), PGI\(_2\), PGE\(_2\), PGF\(_2\alpha\) or PGD\(_2\) by a range of enzymes, many of which remain poorly characterized.

PGD\(_2\) represents a well-characterized eicosanoid that is synthesized in both the central nervous system and peripheral tissues [3,4]. Physiological functions ascribed to this PG include maintenance of body temperature, prevention of platelet aggregation, promotion of sleep, relaxation of smooth muscle, bronchoconstriction and regulation of nerve cell function [5,6]. In addition to these established physiological roles of PGD\(_2\), recent reports [7,8] also identify this eicosanoid as a key mediator of allergic asthma and tactile pain. Whilst the above effects are likely to be mediated predominantly through a specific cell surface G-protein-coupled receptor [9], PGD\(_2\) also represents the precursor of the J\(_2\) series of PGs that serve as activating ligands for the nuclear hormone receptor, peroxisome proliferator-activated receptor-\(\gamma\) [10,11]. Synthetic ligands for this receptor modulate adipocyte differentiation and are used therapeutically as insulin-sensitizing drugs [12].

The isomerization of PGH\(_2\) to PGD\(_2\) is catalysed by PGD\(_2\) synthase (PGDS) enzymes. Two genetically distinct synthases have been identified in human, mouse and rat [13] that have presumably arisen as a consequence of convergent evolution. These isoenzymes are named according to their differential requirement for GSH as a cofactor. The GSH-independent

Abbreviations used: AA, arachidonic acid; CDNB, 1-chloro-2,4-dinitrobenzene; COX, cyclooxygenase; DTT, dithiothreitol; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G-site, GSH-binding site; GST, glutathione S-transferase; PG, prostaglandin; H-site, hydrophobic second substrate (PGH\(_2\))-binding site; IPTG, isopropyl \(\beta\)-D-thiogalactoside; Oct-1, octamer-binding protein-1; ORF, open reading frame; PGDS, prostaglandin D\(_2\) synthase; rPGDS, chicken GSH-dependent PGDS; hPGDS, human GSH-dependent PGDS; mPGDS, mouse GSH-dependent PGDS; rPGDS, rat GSH-dependent PGDS; RT, reverse transcriptase.

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PGDS is a member of the lipocalin superfamily [13], whilst the GSH-dependent PGDS is the only vertebrate member of the class Sigma family of glutathione S-transferases (GSTs) identified to date. The GSTs comprise two distinct superfamilies of enzymes, designated cytosolic and microsomal GST, both of which catalyse conjugation, peroxidation and isomerization reactions with diverse endogenous and exogenous substrates [14,15]. Mammalian cytosolic GSTs have been separated into eight classes (Alpha, Kappa, Mu, Omega, Pi, Sigma, Theta and Zeta) on the basis of their sequence identities [14,16]. Whilst a number of Alpha, Mu and Pi class GST isoenzymes metabolize PGH$_1$ to a mixture of PGE$_2$, PGE$_3$ and PGF$_2$ [17–19], the lack of specificity in these reactions has raised questions about the biological significance of GSTs in PGD$_2$ synthesis. Such a role for GSTs in the synthesis of this eicosanoid became more tenable through the demonstration that a specific GSH-dependent PGDS isolated from rat spleen represented the first vertebrate member of the class Sigma family of GST to be identified [20].

Class Sigma GST family members are widely distributed in nature and include the cephalopod lens S-crystallins [21], the 28 kDa surface antigens from Schistosoma mansoni and Schistosoma japonicum [22], a transferase from the squid Loligo vulgaris [21] and GST isoenzymes from the parasitic helminths Onchocerca volvulus [23], Ascaris suum [24] and Ascardia galli [25]. In addition, the specific GSH-dependent PGDS enzymes that have recently been identified in chicken [26], human and mouse [27] represent vertebrate class Sigma GST members. As this class of transferase includes proteins that possess a uniquely specific PGDS activity, their structure–function properties are of particular interest.

Characterization of the chicken (Gallus gallus) GSH-dependent PGDS (cPGDS) provided initial insights into the catalytic properties of vertebrate class Sigma GSTs [26]. The recombiantly expressed enzyme was found to catalyse the conjugation of GSH with a wide range of aryl halides, organic isothiocyanates and $\alpha,\beta$-unsaturated carbonyls. It was also shown to exhibit low GSH-peroxidase activity towards cumene hydroperoxide. These data suggest that the chicken enzyme may have acquired the ability to perform a role in both detoxification and PGD biosynthesis. Further support for this premise was obtained through examination of its tissue-specific expression. In the chicken, the GSH-dependent PGDS is expressed at highest levels in tissues that are associated with detoxification, such as liver, kidney and intestine. It remains unclear whether mammalian GSH-dependent PGDS enzymes exhibit this potential dual functionality with respect to PGD synthesis and detoxification.

The present study describes the characterization of the catalytic properties of human and rat GSH-dependent PGDS enzymes (hPGDS and rPGDS, respectively). The tissue-specific expression of the enzyme has also been examined in each species. The current investigation, coupled with data generated previously for the orthologous chicken transferase [26], demonstrates that, whilst the PGDS activity of vertebrate class Sigma GSTs appears to be conserved, individual isoenzymes exhibit marked variation with respect to their ability to catalyse the conjugation of GSH with xenobiotics, and their tissue-specific expression.

**MATERIALS AND METHODS**

**Chemicals and enzymes**

Unless stated otherwise, all chemicals were obtained from Sigma–Aldrich (Poole, Dorset, U.K.). Allyl isothiocyanate, benzyl isothiocyanate and 1-iodo-2,4-dinitrobenzene were obtained from Fluka (Poole, Dorset, U.K.). Immobilon-P was obtained from Millipore (Watford, Herts., U.K.). Restriction endonucleases were from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.).

**Antibodies**

Antisera raised against the rPGDS have been described previously [26].

**Isolation of cDNA clones**

A cDNA clone encoding the hPGDS was supplied by the U.K. Human Genome Mapping Project Resource Centre (Hinxton, Cambs., U.K.) [28] in the pT7T3-Pac vector. This expressed sequence tag (EST) clone (clone identity, IMAGE 1758501) was derived from human placental RNA.

A cDNA encoding the rPGDS was isolated from a λgt11 rat spleen 5'-Stretch cDNA library (Clontech, Basingstoke, Hants., U.K.) using a 500 bp fragment of the cPGDS (G. gallus) open reading frame (ORF) as a probe [26]. The ORF of the rat cDNA was amplified by PCR using oligonucleotide primers with the following sequences (restriction sites are underlined): 5'-CAATCATACGAAATCTACATAGCATAGAGG-3' (sense) and 5'-CAAAGGAAAGCTTACACAGCAAGACAC-3' (antisense). The reaction mixture (100 µl) contained 20 mM Tris/HCl, pH 8.8, 2 mM MgSO$_4$, 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 0.1 % (v/v) Triton X-100, 0.1 mg/ml nuclelease-free BSA, each dNTP at a final concentration of 0.2 mM, 50 pmol of each primer, 100 ng of plasmid template DNA and 2.5 units of pfu DNA polymerase (Stratagene, Cambridge, Cambs., U.K.). The amplification reaction was carried out in a Hybrid omnigene thermal cycler (Ashford, Middlesex, U.K.). The template DNA was denatured initially through incubation at 94 °C for 2 min. The ORF was then amplified over 30 cycles through denaturation at 94 °C for 1 min, primer annealing at 50 °C for 1 min, followed by extension at 72 °C for 1 min. The reaction was completed with a single incubation at 72 °C for 5 min. The resulting PCR product was digested with EcoRI and XhoI and subcloned into pBluescript SK$^{-}$ (Stratagene).

**Sequence analysis**

Sequence analysis was carried out by Dr Andrew Cassidy (DNA Sequencing Laboratory, Department of Molecular and Cellular Pathology, Ninewells Hospital, University of Dundee, Scotland, U.K.). The sequence of IMAGE clone 1758501 was determined using M13 forward and reverse priming sites present within pT7T3-Pac vector, whilst the sequence of the rPGDS cDNA clone in pBluescript SK$^{-}$ was determined using the T7 and T3 priming sites present within the vector.

The predicted amino acid sequences of the proteins encoded by these ORFs were determined and analysed using the BIOSOFT GeneJockey II sequence analysis software (Cambridge, Cambs., U.K.). Multiple sequence alignments were performed using thePILEUP program within the GCG Wisconsin package version 8.1 software. The BOXSHADE program (http://www.ch.embnet.org/software/BOX-form.html) was used to display sequence alignments.

**Heterologous expression and protein purification**

The ORF of the hPGDS and rPGDS enzymes was amplified by PCR as described above using pairs of oligonucleotide primers with the following respective sequences (restriction sites are underlined): 5'-CAGAATTGCCATATGCAAAACTACAACA-3' (sense), 5'-GAAGAAAAAACAGCTGAAAGGCCAACATGG-3' (antisense) and 5'-GAACCTGTCATATGCCCAACTACAACCTGC-3' (sense), 5'-TAGTGCTCTCAGAAGTTT-
GCAGATGCTG-3′ (anti-sense). These primers were designed to incorporate NdeI/HindIII and NdeI/XhoI restriction sites into the 5′ and 3′ ends, respectively, of the human and rat cDNAs. The resulting PCR products were digested with the appropriate restriction enzymes and subcloned into the pET17b expression vector (Novagen, Madison, WI, U.S.A.). The fidelity of the PCR was confirmed through direct sequencing of each insert in pET17b from T7 and T3 priming sites.

Recombinant plasmids were used to transform *Escherichia coli* strain BL21. Transformed colonies were grown to exponential phase at 37°C in Luria–Bertani medium containing ampicillin (50 μg/ml), and expression from the pET17b vector was then induced for 2 hr at 37°C through the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG). Bacteria were then harvested by centrifugation at 6000 g for 20 min at 4°C and the cell pellets stored at −70°C until required. Cell pellets derived from 100 ml of induced cultures were resuspended in 20 ml of PBS, pH 7.3, containing 50 μg/ml lysozyme. The cell suspension was incubated at 37°C for 15 min and snap-frozen in liquid N₂. The samples were then defrosted rapidly and sonicated (three separate bursts of 20 s, each of 16 μm amplitude). The insoluble cell debris was removed by centrifugation at 15000 g for 20 min at 4°C. The resulting supernatant was retained and filtered (0.45 μm pore size) under vacuum. The rPGDS was purified on a glutathione–agarose affinity column using the method described previously to isolate the orthologous chicken enzyme [26]. A modified procedure was used to purify the recombinant hPGDS. Briefly, the soluble bacterial lysate containing hPGDS was applied at 0.25 ml/min to a 1.6 cm x 8.0 cm column of glutathione–agarose, previously equilibrated with PBS containing 1 mM dithiothreitol (DTT). The column was then washed with 90 ml of PBS containing 1 mM DTT prior to elution of the bound protein with 10 mM GSH in 100 mM Tris/HCl, pH 8.0. Each purified protein was dialysed for 18 hr at 4°C against two changes (each of 2 l) of 50 μM sodium phosphate, pH 7.0. Dialysed proteins were collected, and glycerol was added to a final concentration of 10% (v/v) prior to storage at −70°C.

**Preparation of rat tissue cytosols**

Wistar rats were obtained from Clare Hall Laboratories (Potters Bar, Herts., U.K.) and killed by cervical dislocation. Organs were removed and frozen immediately in liquid N₂ prior to storage at −70°C. Portions of frozen rat tissues (100 mg) were allowed to thaw in 4 vol. of homogenization buffer (100 mM sodium phosphate, pH 7.0, and 1 mM DTT) and soluble extracts prepared by homogenization with an Omni EZ Connect Homogenizer (Omni International, Gainesville, VA, U.S.A.). Centrifugation at 15000 g for 30 min at 4°C was used to remove cellular debris and the soluble fraction was retained for further study.

**Biochemical analyses**

Protein concentrations were determined according to the method of Bradford [29] with reagents purchased from Bio-Rad (Hemel Hempstead, Herts., U.K.).

GST and PGDS enzyme activities were determined using standard assay conditions as described previously [26]. The $K_{m}$ value for GSH, with respect to GST activity, was determined with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate.

Discontinuous SDS/PAGE was performed according to the method of Laemmli [30] using 12% (w/v) polyacrylamide resolving gels. Equal protein loading was confirmed by staining resolved gels with Coomassie Brilliant Blue R250. For Western-blot analyses, proteins resolved by SDS/PAGE were transferred to Immobilon-P as described previously [31]. Equal protein transfer across the blot was established by staining membranes with Ponseau S. Membranes were incubated with an antibody raised against rPGDS and binding of the immobilized proteins was detected using a goat anti-(rabbit IgG) antibody conjugated with horseradish peroxidase (Bio-Rad) and enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.).

**Molecular modelling**

The crystal structure of the rPGDS [32] was used as a template to generate a model for the hPGDS. A suite of 30 hPGDS models was built using MODELLER (version 4) [33]. The class Sigma GST sequence alignment and subunit I from the Protein Data Bank (PDB entry IPD2) were used as input. The model possessing the lowest energy was then used for comparison against the rPGDS template. The GSH molecule was maintained in the active site throughout the model building process.

**TagMan® quantitative reverse transcriptase (RT)-PCR**

Human tissue or RNA was purchased (Biochain, San Leandro, CA, U.S.A.; Invitrogen, Leek, Netherlands; Clontech, Palo Alto, CA, U.S.A.) or was donated with appropriate ethical approval (Netherlands Brain Bank, Amsterdam, The Netherlands). Poly(A+) RNA was prepared by the PolyATract® system (Promega, Madison, WI, U.S.A.) according to the manufacturer’s instructions. Poly(A+) RNA samples from body tissues isolated from four individuals were quantitated spectrophotometrically or using the RiboGreen® fluorescent method (Molecular Probes, Eugene, OR, U.S.A.), and 1 μg of each RNA was reverse transcribed using random nonamers and Superscript® II RT according to the manufacturer’s instructions (Life Technologies, Rockville, MD, U.S.A.). These cDNAs were then diluted to produce up to 1000 replicate 96-well plates using Biomek Robotics (Beckman Coulter, High Wycombe, Herts., U.K.), such that each well contained the cDNA produced from 1 ng of tissue poly(A+) RNA. The 96-well plates were stored at −80°C prior to TaqMan® mRNA analysis. Relative quantification of mRNA transcripts was performed using the PCR-based 5′ nuclease assay [34] with a gene-specific TaqMan® oligonucleotide probe [35,36]. In order to generate a fluorescent signal upon cleavage by Taq polymerase, each probe was labelled at the 5′-end with the fluorescent reporter dye 6-carboxyfluorescein, and at the 3′-end with the quencher dye 6-carboxy-tetramethylrhodamine via a linker group (PerkinElmer, Foster City, CA, U.S.A.). Primers and probes were designed using Primer Express™ software version 1.0 (PerkinElmer). Oligonucleotide sequences were as follows: GSH-dependent PGDS forward primer 5′-AGATGGTCAATGAGCCTCAGC-3′, GSH-dependent PGDS reverse primer 5′-CCCATGAGAATCTGGCCCAATTAC-3′, GSH-dependent PGDS probe 5′-AA-TGCGGCTTCCATTTGAACATGCAA-3′; GAPDH forward primer 5′-CCCAGTATTGCTGGGCTCAGC-3′, GAPDH reverse primer 5′-GGGCCTTCCACAGTTCTTCC-3′, GAPDH probe 5′-ACCACGTCATCCATGACACTTTC-3′. The relative levels of GSH-dependent PGDS and GAPDH cDNAs within the replicate tissue cDNA 96-well plates were determined by PCR. The amplification mixture containing TaqMan® buffer, uracil-N-glycosylase, 6 mM MgCl₂, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dUTP, 0.2 mM dCTP, each primer at 0.4 μM, 0.1 μM TaqMan® probe and 0.625 units of AmpliTaq Gold® DNA polymerase (PerkinElmer) was transferred using Biomek Robotics (Beckman Coulter), and the plates were sealed with
optical caps (PerkinElmer). Thermal cycling and real-time fluorescence detection were performed with an ABI PRISM™ 7700 Sequence Detection System (PerkinElmer) using the following PCR parameters: 50°C for 2 min, 95°C for 10 min and 45 cycles of 94°C for 15 s, and 60°C for 1 min. The level of mRNA-derived cDNA in each sample was calculated from the TaqMan® signal using plasmid DNA standards included in each PCR plate. The level of genomic DNA contaminating the original RNA samples was found to be negligible (< 10 copies genomic DNA/ng RNA).

RESULTS
Molecular cloning, heterologous expression and purification of GSH-dependent PGDS enzymes

Clones encoding hPGDS and rPGDS enzymes were obtained from placental and spleen cDNA libraries, respectively. The predicted amino acid sequence of the rat enzyme was found to be identical with the previously published sequence [32]. The predicted amino acid sequence of the human synthase encoded by the EST cDNA clone differed from the previously reported sequence [27], in that it contained isoleucine rather than valine at amino acid residue 187. Significantly, Ile<sup>187</sup> is found in the orthologous chicken, mouse and rat enzymes [26, 27, 32].

The molecular masses of hPGDS and rPGDS were calculated to be 23359 Da and 23298 Da, respectively. The amino acid composition of each synthase is similar (Table 1), resulting in an almost identical frequency of acidic, basic, uncharged and non-polar residues. The isoelectric points of hPGDS and rPGDS were thus calculated to be similar, at 5.74 and 5.86, respectively. By contrast, the amino acid composition of the orthologous cPGDS was found to exhibit more obvious differences, resulting in a calculated isoelectric point of 7.37. Alignment of the predicted primary structure of hPGDS and rPGDS also revealed striking similarities between the two synthases (Figure 1). At the protein level, the overall sequence identity between hPGDS and rPGDS was found to be 82%. Catalytically important residues in the rPGDS have previously been identified through crystallography [32] and site-directed mutagenesis [37]. These studies reveal that the sequence identity between hPGDS and rPGDS is particularly pronounced within the GSH-binding site (G-site) and hydrophobic second substrate (PGH<sub>2</sub>)-binding site (H-site). Indeed, all of those residues shown to form the G-site (Tyr<sup>74</sup>, Phe<sup>*79</sup>, Arg<sup>241</sup>, Trp<sup>84</sup>, Lys<sup>30</sup>, Lys<sup>31</sup>, Ile<sup>32</sup>, Pro<sup>29</sup>, Gln<sup>77</sup>, Ser<sup>78</sup> and Asp<sup>81</sup>) and H-site (Met<sup>11</sup>, Arg<sup>242</sup>, Gly<sup>30</sup>, Arg<sup>241</sup>, Glu<sup>77</sup>, Ile<sup>32</sup>, Asp<sup>81</sup>, Met<sup>29</sup>,...
Ser\textsuperscript{109}, Trp\textsuperscript{132}, Tyr\textsuperscript{135}, Ile\textsuperscript{135}, Cys\textsuperscript{136}, Thr\textsuperscript{139} and Leu\textsuperscript{199}) in rPGDS are conserved in hPGDS.

In order to determine whether hPGDS and rPGDS are functionally identical, both proteins were expressed heterologously in E. coli (Figure 2). High levels of IPTG-inducible hPGDS and rPGDS expression were attained, and the recombinant enzymes were retrieved almost exclusively in the soluble fraction of bacterial lysates. The recombinant proteins were isolated using glutathione–agarose affinity chromatography. Surprisingly, chromatography of the enzymes revealed differences between hPGDS and rPGDS with respect to their affinity for the column matrix. It was found that the human synthase slowly leached from the glutathione–agarose during the standard washing procedure. In order to minimize the loss of hPGDS, the affinity column was washed with the minimum volume of PBS buffer required to elute all of the non-specifically bound proteins (90 ml), whereas the rPGDS affinity column could be washed exhaustively (> 270 ml) without significant loss of the rat synthase. As shown in Figure 2, both hPGDS and rPGDS were successfully recovered from the glutathione–agarose. Both proteins were estimated to comprise subunits of approx. 23 kDa molecular mass.

Table 2  GST activities of hPGDS, rPGDS and cPGDS

<table>
<thead>
<tr>
<th>Substrate</th>
<th>hPGDS (nmol/min per mg of protein)</th>
<th>rPGDS (nmol/min per mg of protein)</th>
<th>cPGDS (nmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aryl halides</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1-Bromo-2,4-dinitrobenzene</td>
<td>8.60 ± 0.02</td>
<td>17.6 ± 0.8</td>
<td>128 ± 6</td>
</tr>
<tr>
<td>CNB</td>
<td>5.06 ± 0.01</td>
<td>9.2 ± 0.3</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>1-Fluro-2,4-dinitrobenzene</td>
<td>44.26 ± 1.56</td>
<td>48.3 ± 4.3</td>
<td>410 ± 14</td>
</tr>
<tr>
<td>1-Iodo-2,4-dinitrobenzene</td>
<td>10.70 ± 0.16</td>
<td>17.9 ± 2.3</td>
<td>116 ± 5</td>
</tr>
<tr>
<td>Organic hydroperoxides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>0.052 ± 0.007</td>
<td>0.35 ± 0.03</td>
<td>0.510 ± 0.025</td>
</tr>
<tr>
<td>i-Butyl hydroperoxide</td>
<td>Not detected</td>
<td>Not detected</td>
<td>0.061 ± 0.002</td>
</tr>
<tr>
<td>Organic isothiocyanates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allyl isothiocyanate</td>
<td>6.81 ± 1.02</td>
<td>10.2 ± 1.1</td>
<td>12.6 ± 1.4</td>
</tr>
<tr>
<td>Benzyl isothiocyanate</td>
<td>6.26 ± 0.42</td>
<td>11.3 ± 1.8</td>
<td>17.6 ± 1.3</td>
</tr>
</tbody>
</table>

Substrate specificity of GSH-dependent PGDS isoenzymes from different species

The ability of hPGDS and rPGDS to catalyse the isomerization of PGH₂ to PGD₂ was verified using a coupled assay in which the PGH₂ substrate was generated in situ from [\textsuperscript{14}C]AA. Analysis of the reaction products by TLC showed that both enzymes converted PGH₂ to PGD₂ (Figure S3). The assay also demonstrated that neither enzyme catalysed the formation of PGE₃ or PGF₃α.

The catalytic specificity of hPGDS and rPGDS was explored using a range of model GST substrates. Table S2 shows that the human and rat enzymes exhibited activity towards the same range of substrates as the previously described chicken syn-
Table 3 Kinetic parameters of hPGDS and rPGDS enzymes

The $K_m$ value for GSH of the hPGDS with respect to GST activity was determined in the present study. All other values were taken from Kanaoka et al. [27].

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>hPGDS</th>
<th>rPGDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGDS activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ for PGH$_2$ (mM)</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>$K_m$ for GSH (mM)</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>GST activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ for CDNB (mM)</td>
<td>3.2</td>
<td>5.0</td>
</tr>
<tr>
<td>$K_m$ for GSH (mM)</td>
<td>8.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Both hPGDS and rPGDS exhibited highest transferase activity towards aryl halides and organic isothiocyanates. They were also found to possess limited ability to catalyse the reduction of cumene hydroperoxide. Under standard assay conditions, hPGDS and rPGDS exhibited substantially lower specific activities towards aryl halides than cPGDS. For example, hPGDS and rPGDS demonstrated specific activities towards CDNB of 5.1 and 9.2 $\mu$mol/min per mg of protein, respectively, whereas under identical conditions cPGDS gave a value of 97.0 $\mu$mol/min per mg of protein. Significantly, despite a high level of sequence identity, Table 2 also demonstrates that the human synthase exhibited considerably lower transferase activity towards a number of aryl halides than the rat enzyme. In particular, the specific activity of hPGDS towards 1-bromo-2,4-dinitrobenzene, CDNB and 1-iodo-2,4-dinitrobenzene was only approx. 50% of that exhibited by rPGDS.

The transferase activity of hPGDS and rPGDS towards organic isothiocyanates did not differ so markedly from that of cPGDS.

GSH-dependent PGDS isoenzymes display marked variation in their kinetic properties

In order to understand the reason for the differences in the affinity of hPGDS and rPGDS for glutathione-agarose, and their different specific activities towards aryl halides, the kinetic properties of each enzyme was examined [27,38]. Table 3 shows that, although the human and rat enzymes exhibit similar substrate $K_m$ values with respect to both PGDS activity and

Figure 4 Ribbon diagram of the N-terminal domain of hPGDS and rPGDS

Ribbon diagrams of the N-terminal domain of the rPGDS crystal structure (a) and the hPGDS structure (b) are shown complexed with GSH. Those residues responsible for binding GSH are shown in cyan. Those illustrated include the potentially catalytically important Tyr$^8$ residue (interaction with the GSH thiol moiety indicated by a white dotted line), and the glycyl carboxylate complexing residues, Trp$^{39}$ and Lys$^{43}$ (interaction with the glycyl carboxylate moiety indicated by a white dotted line). The magenta coloured sites are the positions of variance between the hPGDS and rPGDS enzymes. The substitutions with possibly the greatest effect on GSH binding are shown in blue in the hPGDS model. Other residues illustrated include Ala$^{37}$ and Pro$^{40}$. 
Cytosol extracts from rat organs were separated by SDS/PAGE in 12% (w/v) resolving gels. Electrophoretically resolved proteins were transferred to an Immobilon-P membrane and immunoblotted with a polyclonal antibody raised against the rPGDS. The gel was loaded with 2 ng of purified recombinant rPGDS (standard) and 10 μg of tissue cytosol extracts. Resolved gels and blotting membranes were stained to confirm equal protein loading and uniform protein transfer.

Transf erase activity, marked differences were apparent with respect to their $K_m$ for GSH in a standard conjugation reaction using CDNB as substrate. The $K_m$ for GSH determined for the human synthase in this reaction was found to be 25-fold higher than that reported previously for the rat enzyme (8 mM versus 0.3 mM, respectively) [27].

Molecular basis for the differences in catalytic properties between hPGDS and rPGDS

hPGDS and rPGDS are predicted to share 82% amino acid sequence identity. Furthermore, the active-site residues appear to be identical. In the absence of any G-site or H-site heterogeneity between the two synthases, a molecular modelling approach was adopted to identify amino acid substitutions outwith these regions, which may account for the different $K_m$ values for GSH exhibited by these enzymes. The crystal structure of the rat synthase [32] was used to generate a model structure for hPGDS. A ribbon diagram showing the tertiary structure of the N-terminal domain (residues 1–70) of each protein is shown in Figure 4. The two enzymes exhibit 92% sequence identity over this region, and all of the rat G-site residues are conserved in hPGDS. GSH is thus predicted to bind the human and rat G-sites in a similar orientation. The N-terminal domain of the rat synthase is composed of a four-stranded $\beta$-sheet and three $\alpha$-helices. There are six amino acid differences between rPGDS and hPGDS within this N-terminal domain (Leu$^7$ → Thr, Lys$^{28}$ → Gln, Lys$^{41}$ → Glu, Pro$^{44}$ → Ser, Val$^{53}$ → Ile and Glu$^{57}$ → Asp, for rPGDS and hPGDS, respectively). Analysis of the model hPGDS structure indicates that certain of these substitutions may influence, through indirect or second sphere effects, the interaction between GSH and the G-site. In particular, the Lys$^{41}$ → Glu substitution in hPGDS is predicted to eliminate a potential hydrogen bond between the Lys$^{41}$ Nz and the Ala$^{57}$ main chain carboxyl group. The concurrent change of Pro$^{44}$ to Ser in hPGDS may also cause a modification of the C-terminus of $\alpha$-helix 2. Either, or both, of these substitutions may elicit a second sphere effect on GSH binding through modification of the interaction of Trp$^{28}$ and Lys$^{41}$ with the glycol carboxylate moiety. We propose that these amino acid substitutions are predominantly responsible for the differences in the catalytic properties of the two mammalian synthases.

**Figure 5** Tissue-specific expression of GSH-dependent PGDS in the rat

Relative levels of both GSH-dependent PGDS and GAPDH mRNA in RNA samples isolated from various human tissues were determined by TaqMan® quantitative RT-PCR. Poly(A)$^+$ tissue RNA samples from four individuals were reverse transcribed using random nonamers. Relative quantification of mRNA-derived cDNA was then achieved using the PCR-based 5'-nuclease assay with a fluorescently labelled gene-specific oligonucleotide probe and real-time fluorescence detection. Results for hPGDS (solid bars) are expressed as mean mRNA-derived cDNA $\times 10^{-3}$, whereas GAPDH results (open bars) are expressed as mean mRNA-derived cDNA $\times 10^{-4}$. All values are means ± S.E.M. for RNA samples from four individuals. PBMC, peripheral blood mononuclear cells.

**Tissue-specific expression of GSH-dependent PGDS enzymes**

Inter-species variation in the catalytic properties of the synthases suggested heterogeneity in their physiological functions. We addressed this possibility by determining tissue-specific expression profiles in both hPGDS and rPGDS. The relative expression levels of the enzyme in rat tissues was determined by Western blotting (Figure 5). The highest level of PGDS protein was detected in rat spleen and bone marrow, with lower levels present in small intestine, colon, liver, pancreas and skin. Expression of...
the synthase could not be detected in rat brain, heart, lung or kidney. We next determined the relative levels of PGDS mRNA in human tissues by TaqMan® quantitative RT-PCR (Figure 6). The expression profile obtained for the human synthase was markedly different. In particular, whilst PGDS expression was enriched in rat bone marrow and spleen, mRNA for the enzyme was virtually undetectable in the corresponding human tissues. Conversely, whilst moderate levels of PGDS expression were detected in human lung, the synthase could not be detected in rat lung. Those human tissues or cell types in which the PGDS was expressed at highest levels include adipose, macrophages and placenta.

DISCUSSION

The only vertebrate members of the class Sigma family of GSTs identified to date are the hPGDS, rPGDS, cPGDS and mouse GSH-dependent PGDS (mPGDS) enzymes. Whilst possessing detoxification activities characteristic of other members of the two GST superfamilies, these enzymes also have the unique ability to catalyse specifically the isomerization of PGH₂ to PGD₂. Production of PGD₂ is a physiologically important reaction and, therefore, the human synthase represents a potential drug target site.

It was previously unclear whether class Sigma GST exhibit similar catalytic properties. The present study describes a direct comparison of two mammalian PGDS enzymes that has revealed key differences in the catalytic properties of the human and rat synthases. Furthermore, the basis for these differences is proposed through molecular modelling.

PGDS activity of class Sigma GSTs

With respect to PGDS activity, a $K_m$ value of 200 $\mu$M for PGH₂ has been obtained previously for each of the hPGDS, rPGDS and mPGDS enzymes [27]. These enzymes also exhibit similar $K_m$ values for GSH with respect to PGDS activity [27], demonstrating that the catalytic properties of the synthases are comparable in this regard. Characterization of these enzymes has revealed that all of the H-site and adjacent amino acid residues are conserved in the mammalian enzymes, consistent with their similar PGDS activities. The cPGDS also shares substantial H-site sequence identity with the mammalian synthases. In the avian enzyme, 11 of the 15 defined H-site residues are retained (Arg12, Gly13, Arg14, Glu16, Ile17, Asp18, Met19, Trp101, Tyr102, Cys104 and Thr109) and three of the remaining residues (amino acids 11, 155 and 199) are represented by conservative substitutions. The single non-conservative H-site substitution in cPGDS is Ser100 to Met. The H-site heterogeneity in the avian enzyme is accompanied by an increased $K_m$ for PGH₂ [39]. By contrast, the squid class Sigma GST has the ability to convert PGH₂ to a mixture of PGD₂, PGE₂ and PGG₂, and therefore does not exhibit the unique specificity of the vertebrate enzymes [27]. Consistent with these observations, Figure 1 reveals that only three of the 15 PGH₂-binding residues in the rPGDS are conserved in the squid GST.

Catalytic activity of class Sigma GSTs towards aryl halides

The first functional differences between hPGDS and rPGDS became apparent during affinity chromatography when it was found that the human synthase was more readily lost from the column during the washing procedure than was the rat enzyme. Comparison between the specific activities of the human and rat synthases with a number of model GST substrates demonstrated differences in their catalytic properties. Although exhibiting broadly similar substrate specificities, the activity of the human enzyme towards aryl halides was consistently only approx. 50% of that of the rPGDS. Furthermore, both of the mammalian enzymes were substantially less active than the previously characterized chicken synthase.

Analysis of the kinetic parameters of the human and rat enzymes with respect to their transferase activity towards an aryl halide revealed that although the synthases exhibited similar $K_m$ values for CDNB, the corresponding values for GSH in this reaction are markedly different. It is likely that the higher $K_m$ value for GSH exhibited by hPGDS accounts at least in part for the lower specific activities exhibited by the enzyme under standard assay conditions. Interestingly, whilst the mPGDS also exhibits a similar $K_m$ value for CDNB, the corresponding value for GSH lies between those exhibited by the human and rat synthases [27]. It remains to be determined how the specific activities of the mPGDS compare with those obtained for the human, rat and chicken enzymes.

The apparent differences in $K_m$ for GSH amongst the mammalian synthases with respect to conjugation and isomerization reactions is interesting given the presumed interaction of GSH with identical G-site residues in all cases. This disparity is likely to reflect enzyme-specific differences in the spatial arrangement of GSH and substrate, imposed through the structural diversity of GSH and substrate.

Tissue-specific expression of class Sigma GSTs

Marked inter-species variation occurs in the tissue-specific expression of GSH-dependent PGDS enzymes. Previous workers have proposed that, in the rat, the synthase serves a role in haematopoiesis and immune function [32,40-42]. Our Western-blot data are consistent with these hypotheses. In the present study, TaqMan® quantitative RT-PCR has been used to determine the levels of PGDS mRNA in human tissues. Certain aspects of the RT-PCR data are consistent with the synthase possessing similar physiological functions in humans as in the rat. For example, in the human, PGDS is expressed in foetal liver and macrophages. However, significant species differences are evident in the expression profiles in human and rat tissues. Perhaps most striking is the variation in the relative abundance of the enzyme in bone marrow and spleen. Although the level of expression of the synthase in rat bone marrow and spleen is considerably higher than in any other tissue, PGDS mRNA is virtually undetectable in these tissues in humans. These differences occur despite a degree of conservation in the cellular origins of the synthase. Particularly relevant in this context is the fact that PGDS expression has been observed in immune cells derived from mouse [43], rat [40-42] and human [44]. It is interesting to note that these differences in tissue-specific expression extend to the chicken and mouse, which also possess low or undetectable levels of PGDS in spleen and bone marrow [26,27]. In the case of the former, highest levels of expression were observed in kidney, liver, pancreas and intestine, whereas skin was the only tissue in which PGDS expression was detected in mouse. It should be emphasized that the absence of the GSH-dependent PGDS may not necessarily exclude a functional role for PGD₂ in certain tissues. In humans, the GSH-independent PGDS is expressed at high levels in brain and heart (results not shown), and may therefore compensate for the low levels of GSH-dependent PGDS gene expression observed in these tissues.

Although the molecular basis for these differences in tissue-specific expression have yet to be elucidated, analysis of the hPGDS gene promoter helps rationalize certain aspects of
the expression profile [45]. Potential regulatory elements identified include an octamer-binding protein-1 (Oct-1) element, five GATA elements, two polynucle enhancer activator 3 (PEA3) elements, one cAMP-response element (CRE)-like sequence and one activator protein-2 (AP-2) element. It is interesting to note that a number of these elements have been associated with placenta-specific and lymphoid-specific gene expression. In particular, both an Oct-1 binding site and a GATA-like element are found in the placenta-specific enhancer of the human leukaemia inhibitory factor receptor gene [46]. Such binding sites have also been identified in the promoters of a number of lymphocyte-specific genes [47,48]. The extent to which these regulatory elements contribute to the expression profile of the PGDS in human tissues has yet to be established. The rPGDS gene promoter has not been characterized to date.

Endogenous roles of vertebrate class Sigma GSTs

The different catalytic properties of the hPGDS and rPGDS enzymes, coupled with their divergent tissue-specific expression profiles, reflect distinct endogenous roles for these enzymes. Whilst the kinetic data available suggest that the chicken, human and rat synthases all contribute to PGD₃ production in vitro, our data suggest that the same may not be true for their putative detoxification activities, which involve GSH conjugation. In a typical conjugation reaction, a \( K_m \) value for GSH of approx. 8 mM was determined for the human synthase, in comparison with a value of 0.3 mM previously reported for the rat enzyme [27]. It is thus probable that the conjugating activity of hPGDS will be limited by the availability of GSH. It has also been demonstrated that certain class Alpha, Mu and Pi GSTs, which exhibit low \( K_m \) values for GSH (50–200 \( \mu M \)), are subject to product inhibition, and may therefore serve to sequester and transport GSH conjugates within the cell. [49]. These observations imply that the relatively high \( K_m \) value for GSH exhibited by the human enzyme may limit product inhibition by aryl halides. It is thus unlikely that hPGDS contributes to the sequestration and transport of such xenobiotics. Our data therefore suggest that the human synthase may have evolved a highly specific PGDS function at the expense of the general transferase activity exhibited by other GST. The catalytic data generated previously for the chicken synthase suggest that this enzyme may represent a more primitive vertebrate member of the class Sigma family of GST, which has retained high levels of activity towards model substrates whilst acquiring PGDS activity. The contribution of vertebrate class Sigma GST to detoxification reactions in vivo is thus likely to vary considerably between species. From the available data we propose that the magnitude of this contribution is progressively reduced from chicken to rat and from rat to human.

The tissue-specific expression data support the hypothesis that PGDS plays a variable role in the detoxification of xenobiotics. We have previously shown [26] that the chicken synthase is expressed predominantly in liver, kidney and intestine; all tissues in which detoxification is of physiological importance. In human and rat, the synthase is not expressed in organs generally associated with detoxification. Rather, the expression profile of PGDS in these species correlates well with tissues or cell types in which a role for PGD₃ has been established. This is perhaps best exemplified through the expression of the synthase in cells or organs associated with immune function. The relevance of expression in other tissues can also be hypothesized. In particular, the demonstration that PGDS expression is enriched in human adipose tissue is consistent with the notion that PGD₃-derived eicosanoids may represent a group of endogenous ligands for the pro-adipogenic transcription factor, peroxisome proliferator-activated receptor-\( \gamma \).

Concluding remarks

The present study describes the characterization of hPGDS and rPGDS enzymes. The data presented concerning the catalytic properties and tissue-specific expression of the synthases raise important questions about the physiological function and evolutionary relationship between class Sigma GST family members. The extent to which either the PG biosynthetic activity of class Sigma GSTs can be assumed by GSH-independent PGDSs, or the detoxification function of class Sigma GSTs can be assumed by other GST isoenzymes, remains to be established. Such characterization of these enzymes will aid the rational design of specific PGDS inhibitors which may be of therapeutic benefit in the treatment of conditions associated with inappropriate PGD₃ synthesis, such as mastocytosis and allergic asthma.

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