Sequence, catalytic properties and expression of chicken glutathione-dependent prostaglandin D_2 synthase, a novel class Sigma glutathione S-transferase

Anne M. THOMSON*, David J. MEYER† and John D. HAYES*[1]

*Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland, U.K., and †Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K.

The Expressed Sequence Tag database has been screened for cDNA clones encoding prostaglandin D_2 synthases (PGDSs) by using a BLAST search with the N-terminal amino acid sequence of rat GSH-dependent PGDS, a class Sigma glutathione S-transferase (GST). This resulted in the identification of a cDNA from chicken spleen containing an insert of approx. 950 bp that encodes a protein of 199 amino acid residues with a predicted molecular mass of 22732 Da. The deduced primary structure of the chicken protein was not only found to possess 70% sequence identity with rat PGDS but it also demonstrated more than 35% identity with class Sigma GSTs from a range of invertebrates. The open reading frame of the chicken cDNA was expressed in *Escherichia coli* and the purified protein was found to display high PGDS activity. It also catalysed the conjugation of glutathione with a wide range of aryl halides, organic isothiocyanates and α,β-unsaturated carbonyls, and exhibited glutathione peroxidase activity towards cumene hydroperoxide. Like other GSTs, chicken PGDS was found to be inhibited by non-substrate ligands such as Cibacron Blue, haematin and organotin compounds. Western blotting experiments showed that among the organs studied, the expression of PGDS in the female chicken is highest in liver, kidney and intestine, with only small amounts of the enzyme being found in chicken spleen; in contrast, the rat has highest levels of PGDS in the spleen. Collectively, these results show that the structure and function, but not the expression, of the GSH-requiring PGDS is conserved between chicken and rat.

INTRODUCTION

Prostaglandins (PGs) are a widely distributed group of oxygenated eicosanoids that are involved in the control of various defence and homeostatic mechanisms in the body [1]. They are derived from polyunsaturated fatty acids by the activity of cyclo-oxygenase (COX) to produce the intermediate PGH_2 [1]. Two COX isoenzymes, COX-1 and COX-2, have been identified and shown to be differentially regulated. COX-1 is expressed constitutively in most mammalian cells, whereas COX-2 is normally present in smaller amounts but is inducible by cytokines, mitogens, serum and endotoxin [2–4]. The PGH_2 produced from arachidonic acid is a short-lived species and can be converted into PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 or thromboxane A_2 by the actions of specific synthases [1,5]. PGD_2 is a major eicosanoid generated by a number of tissues [6,7]. It has been recognized for many years that PGD_2 is involved in diverse physiological processes such as maintenance of body temperature, prevention of platelet aggregation, promotion of sleep, relaxation of smooth muscle, bronchoconstriction and nerve cell function [8,9]. PGD_2 is the most abundant prostanoid produced by mast cells, and upon mast cell activation it is believed to function as an allergic and inflammatory mediator [10]. In addition to these biological roles for PGD_2, which in large part are effected through cell surface receptors, this eicosanoid is also of importance because it gives rise to the cyclopentenone-type J_2 series of PGs that have their own characteristic spectrum of effects [11]. PGD_2 readily undergoes dehydration both *in vitro* and *in vivo* to yield PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 [12].

The isomerization of PGH_2 to PGD_2 is catalysed by PGD_2 synthase (PGDS) [5]. Two genetically distinct forms of the synthase have been described, namely the GSH-dependent and GSH-independent PGDS isoenzymes [13,14]. The two rat PGDS isoenzymes have also been given designations according to their tissue of origin: the GSH-dependent enzyme has been called the spleen-type PGDS or the haemopoietic PGDS, whereas the GSH-independent enzyme has been called brain PGDS. The GSH-dependent PGDS in the rat has recently been identified as a class Sigma member [15] of the glutathione S-transferase (GST) superfamily [16]. GSH-dependent PGDS was first isolated from rat spleen by Christ-Hazelhof and Nugteren [17]. More recent purification of the PGDS from rat spleen allowed its biochemical and immunochemical characterization [18] and led to the demonstration that the expression of this enzyme in the rat is restricted primarily to mast cells and antigen-presenting cells including histiocytes, dendritic cells and Kupffer cells [19,20].

Although GSH-dependent PGDS in the rat was the first mammalian member of the class Sigma GST to be characterized, this transferase family is widely distributed in Nature. The class Sigma family was first defined by Buetler and Eaton [21] by using sequence alignments of cDNA clones for S-crystallins and GST from molluscs [22,23] (see also [24]), as well as cDNA clones for surface antigens from parasitic helminths [25]. The catalytic properties of class Sigma GST are poorly described, possibly

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; COX, cyclo-oxygenase; EST, expressed sequence tag; GST, glutathione S-transferase; ORF, open reading frame; PG, prostaglandin; PGDS, prostaglandin D_2 synthase. In this paper the PGDS isoenzymes are designated in accordance with their requirement for glutathione because this property is unlikely to vary with species, whereas tissue-specific expression can differ from one species to another.

[1] To whom correspondence should be addressed (e-mail hayes@icrf.icnet.uk).

The nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank, GSDB and DDBJ Nucleotide Sequence Databases under the accession number AJ006405.

because certain of them are not readily purified because they fail to bind commercially available glutathione affinity columns [24]. It is not known whether PGDS activity is a general property of class Sigma GSTs, nor is it known whether enzymes of this class typically display a broad specificity towards model transferase substrates. In view of the physiological properties of PGs, it is important to know whether the ability of the rat class Sigma GST to act as a PGH_2:PGD_2 isomerase is a unique property of this enzyme, or whether it is a more common feature shared by members of this class of transferase from other species. Interestingly, the parasitic worm *Ascaridia galli* possesses a class Sigma GST that has been shown to display a high PGH_2:PGE_2 isomerase activity [26]. Furthermore in view of the widespread distribution of class Sigma GSTs and their presence in parasites it would be helpful to establish the types of detoxification reaction that are catalysed by this GST family.

In the present study a cDNA for a chicken GSH-dependent PGDS is described. The catalytic properties of the chicken PGDS towards a range of GST substrates has been determined, as has its tissue-specific expression. The relationship between the chicken PGDS and the previously described chicken hepatic GST CL5 subunit [27] is discussed.

MATERIALS AND METHODS

Chemicals and enzymes

Unless stated otherwise, all chemicals were from Sigma-Aldrich Company Ltd (Poole, Dorset, U.K.). The GST substrate 4-hydroxynon-2-enal was provided by Professor H. Esterbauer (University of Graz, Graz, Austria), 1-menaphthyl sulphate was custom-synthesized by Ultrafine Chemicals (Manchester, Greater Manchester, U.K.), and allyl isothiocyanate, benzyl isothiocyanate, tributyltin acetate and 1-iodo-2,4-dinitrobenzene were from Fluka (Poole, Dorset, U.K.). Immobilon-P was from Millipore (Watford, Herts., U.K.).

All restriction endonucleases were from Gibco BRL Life Sciences (Paisley, Renfrewshire, U.K.). The fmol® DNA Cycle Sequencing System was purchased from Promega (Southampton, Hants., U.K.).

Antibodies

Antisera against the rat GSH-dependent PGDS were raised in female New Zealand White rabbits with an immunization protocol similar to that described previously [28]. Rat PGDS was isolated from frozen (−70 °C) spleen with chromatography methods that have been established for the purification of GST [15,16]. In brief, frozen spleen (approx. 30 g) was allowed to partly thaw at room temperature before being diced manually and homogenized in 5 vol. of ice-cold 20 mM Tris/HCl/200 mM NaCl buffer, pH 7.4, containing 0.5 mM dithiothreitol (buffer A). The homogenate was centrifuged for 1 h at 100000 ***g*** and 4 °C; the resulting supernatant was applied directly to a 4.4 cm × 7.0 cm column of glutathione–agarose pre-equilibrated and eluted (4 °C, 60 ml/h) with buffer A. The column was washed with at least 300 ml of buffer A before the retained GST isoenzymes were eluted with a solution of 20 mM GSH in 200 mM Tris/HCl buffer, pH 9.5. The material recovered from the affinity column was dialysed at 4 °C for a total of 24 h against two changes, each of 2 litres, of 20 mM Tris/HCl buffer, pH 7.8, containing 0.5 mM dithiothreitol (buffer B). Finally, rat PGDS was resolved from other spleen GSTs by anion-exchange FPLC on mono Q (performed at room temperature) pre-equilibrated with buffer B, and developed with a 0–80 mM NaCl gradient formed in the same buffer; by this method, homogeneous rat PGDS was recovered as the last major protein-containing peak to be eluted from mono Q. Immunization was performed with between 30 and 50 μg of the rat PGDS.

Identification of chicken GSH-dependent PGDS cDNA

A chicken (*Gallus gallus*) cDNA encoding a protein with an amino acid sequence related to the 48 N-terminal residues of rat GSH-dependent PGDS was identified by a BLAST search of the Expressed Sequence Tag (EST) database through the National Centre for Biotechnology Information's World Wide Web site (http://www.ncbi.nlm.nih.gov/BLAST/). The EST clone (pat.pk0008.b4) was isolated from a concanavalin A-activated chick splenic T-cell cDNA library constructed by Dr. Joan Burnside (University of Delaware, Newark, DE, U.S.A.). This clone was supplied to us by Dr. Joan Burnside in a pcDNA3 vector.

Both strands of pat.pk0008.b4 in the pcDNA3 vector were sequenced with the Promega fmol® DNA Cycle Sequencing System in accordance with the manufacturer's instructions. Oligonucleotides synthesized to the T7 and SP6 promoter sequences were used to sequence each end of the clone, and sequence-specific oligonucleotides were used to sequence within the insert.

A multiple sequence alignment between the predicted full-length chicken PGDS protein sequence and other related proteins in the SwissProt and GenBank databases, which had been identified by a BLAST search, were aligned with the PILEUP program in the GCG Wisconsin package Version 8.1 software.

Heterologous expression and protein purification

The open reading frame (ORF) of pat.pk0008.b4 was amplified by PCR with 5′-AGGACCCCATATGCCCAACTACAAGC-TGACG-3′ (sense) and 5′-CTAGATTCTGATTTGGTACTC-GAGATCCACC-3′ (anti-sense) to introduce *Nde*I and *Xho*I restriction sites into the 5′ and 3′ ends of the cDNA. The amplification reaction mixture (100 μl) contained 20 mM Tris/HCl buffer, pH 8.0, 10 mM KCl, 10 mM $MgSO_4$, 0.1 % (v/v) Triton X-100, 10 μg/ml nuclease-free BSA, each dNTP at 0.2 mM, 0.15 ng of template DNA, 2.5 units of *pfu* DNA polymerase (Stratagene, Cambridge, Cambs., U.K.) and 50 pmol of each primer. PCR was performed in a Hybaid omnigene thermal cycler with the following programme: cycle 1, 94 °C for 2 min; cycles 2–31, each 94 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min; cycle 32, 72 °C for 6 min. The resulting PCR product was digested with the appropriate restriction enzymes and ligated into the *Nde*I and *Xho*I sites in the bacterial expression vector pET17b (Novagen, Madison, WI, U.S.A.). The cDNA insert in pET17b was sequenced to confirm the fidelity of the PCR reaction.

The chicken clone in pET17b was transformed into *Escherichia coli* BL21 pLysS. Colonies were picked and grown to exponential phase in Luria–Bertani medium containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) before expression from the pET17b vector was induced with 1 mM isopropyl β-D-thiogalactoside. After the transformed cells had been allowed to grow for 2 h in the presence of isopropyl β-D-thiogalactoside, they were harvested by centrifugation (30 min at 15000 ***g***) and cell pellets were frozen at −70 °C until required. The frozen bacterial pellet (from 100 ml of liquid culture) was allowed to thaw at ambient temperature before being resuspended in 20 ml of 50 mM sodium phosphate buffer, pH 7.0 (buffer C), containing 0.01 % (v/v) NP40 detergent. The resuspended cells were snap-frozen in liquid nitrogen, thawed and resuspended in 80 ml of buffer C, before being finally disrupted by 10 min of treatment at

37 °C with lysozyme (sufficient to give 50 μg/ml) and sonication (three separate bursts of 20 s, each of amplitude 16 μm). The insoluble bacterial debris was removed by centrifugation (15000 ***g*** for 20 min at 4 °C) and the resulting supernatant was filtered under vacuum. The soluble lysate (approx. 100 ml) was applied directly to a 1.6 cm × 8.0 cm column of glutathione–agarose that had been equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl (buffer D). This column was eluted at 30 ml/h and, after it had been washed with 100 ml buffer D, was developed with a solution of 10 mM GSH in 200 mM Tris/HCl buffer, pH 9.1. The protein eluted by GSH was collected and dialysed for 18 h at 4 °C against two changes, each of 2 litres, of buffer C containing 1 mM dithiothreitol. The dialysed protein was collected and glycerol was added to a final concentration of 10 % (v/v) before being stored at −70 °C.

Cytosol preparation of chicken tissues

Sexually mature adult hens were purchased from a local Dundee poultry farmer. The hens were killed by a lethal injection of Euthatal, after which organs were removed and frozen immediately in liquid nitrogen before being stored at −70 °C until use.

Portions (approx. 1 g) of frozen chicken tissues were allowed to thaw in 4 vol. of buffer C and soluble extracts were prepared by homogenization with an Omni EZ Connect Homogenizer (Omni International, Gainesville, VA, U.S.A.), followed by centrifugation to remove cellular debris (15000 ***g*** for 30 min at 4 °C).

Protein determination and GST assays

Protein concentrations were measured by the method of Bradford [29] with reagent purchased from Bio-Rad Laboratories (Hemel Hempstead, Herts., U.K.).

All GST enzyme activity assays were conducted at 37 °C. In all instances, the non-enzymic reaction was measured and subtracted from the overall reaction rate. Activity towards the majority of the substrates was determined manually with a Shimadzu UV 3000 spectrophotometer (Haverhill, Suffolk, U.K.) at the following wavelengths and pH conditions: allyl isothiocyanate, 274 nm and pH 6.5; Δ^5-androstene-3,17-dione, 248 nm and pH 8.5; benzyl isothiocyanate, 274 nm and pH 6.5; 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, 419 nm and pH 5.0; *trans*,*trans*-deca-2,4-dienal, 280 nm and pH 7.5; 1,2-dichloro-4-nitrobenzene, 345 nm and pH 7.5; 1,2-epoxy-3-(4′-nitrophenoxy)-propane, 360 nm and pH 6.5; ethacrynic acid, 270 nm and pH 6.5; 4-hydroxynon-2-enal, 230 nm and pH 7.5; 1-menaphthyl sulphate, 298 nm and pH 7.5; 4-nitrobenzyl chloride, 310 nm and pH 6.5; 4-nitrophenyl acetate, 400 nm and pH 7.0; *trans*-non-2-enal, 225 nm and pH 7.5; *trans*-4-phenylbut-3-en-2-one, 290 nm and pH 6.5 [30–34]. Activity towards 1-chloro-2,4-dinitrobenzene (CDNB), 1-bromo-2,4-dinitrobenzene, 1-fluoro-2,4-dinitrobenzene and 1-iodo-2,4-dinitrobenzene, as well as glutathione peroxidase activity with cumene hydroperoxide and t-butyl hydroperoxide were measured at 340 nm with a Cobas Fara II centrifugal analyser (Hoffmann-La Roche Ltd., Basel, Switzerland) [30,35].

Determination of PGDS activity

Measurement of PGDS activity was performed with a coupled enzyme assay. Radiolabelled PGH_2, which was generated *in situ* from [^{14}C]arachidonic acid, served as substrate for PGDS as described by Meyer and Thomas [15]. Reactions were left for 1.6 min at 37 °C before being placed on ice. Reaction products were extracted, separated by TLC with ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (11:5:2:10, by vol.) and individual PGs were quantified by radioactivity scanning (Bioscan; Lablogic, Sheffield, U.K.). The PGDS activity was tested with a final concentration of either 1.3 μg or 13 μg of protein/ml and 0.5 mM GSH.

Electrophoresis and Western blot analyses

Discontinous SDS/PAGE was performed by the method of Laemmli [36] in 12 % (w/v) polyacrylamide resolving gels. For Western blotting analyses, the proteins resolved by SDS/PAGE were transferred to Immobilon-P by methods described previously [37]. Antibodies against rat GSH-dependent PGDS that had adsorbed specifically on to immobilized proteins on the blot were allowed to react with goat anti-(rabbit IgG) antibodies, and the complexes were detected by enhanced chemiluminescence with reagents from Amersham Life Science (Little Chalfont, Bucks., U.K.).

RESULTS

Sequence of cDNA from chicken with sequence similarity to rat GSH-dependent PGDS

A search of the EST database with the N-terminal amino acid sequence of rat PGDS [13,15] revealed the presence of a chicken cDNA (pat.pk0008.b4) encoding a protein sharing 38 of the first 48 residues found in the enzyme purified from rat spleen. This EST, which was supplied to us by Dr. Joan Burnside, was sequenced to determine whether it might represent a chicken PG synthase. Figure 1 shows the sequence of the chicken cDNA clone (approx. 900 bp in length). It contains an ORF of 597 bp for a protein of molecular mass 22732 Da. The clone contains 36 bp upstream from the putative ATG initiation codon and 262 bp

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                                          -36                        -1
                                           gaagggatcacacaccgctgcacccaaggacccatc

M   P   N   Y   K   L   T   Y   F   N   L   R   G   R   A   E   I   C   R   Y       20
ATG CCC AAC TAC AAG CTG ACG TAC TTC AAT CTG CGA GGA AGA GCA GAA ATC TGC CGC TAC     60

L   F   A   Y   A   G   I   K   Y   E   D   H   R   L   E   G   A   D   W   P       40
CTG TTT GCC TAT GCA GGC ATA AAG TAT GAA GAC CAC AGG TTG GAA GGA GCA GAC TGG CCC    120

K   I   K   P   T   I   P   F   G   K   V   P   I   L   E   V   D   G   V   I       60
AAA ATT AAA CCA ACT ATC CCA TTT GGC AAA GTT CCT ATT CTG GAA GTA GAT GGA GTG ATC    180

I   H   Q   S   L   A   I   A   R   Y   L   A   R   E   S   G   L   A   G   Q       80
ATT CAC CAG AGC CTA GCA ATT GCG AGA TAC CTC GCC AGA GAA TCA GGT CTG GCA GGT CAG    240

T   P   V   E   Q   A   L   A   D   A   I   V   D   T   I   D   D   F   M   M      100
ACT CCA GTG GAG CAG GCA TTG GCT GAT GCC ATT GTG GAC ACC ATT GAT GAT TTC ATG ATG    300

L   F   P   W   A   E   K   N   Q   D   V   K   E   K   A   F   N   D   I   L      120
CTG TTC CCC TGG GCA GAG AAA AAT CAG GAT GTG AAG GAG AAA GCT TTT AAT GAC ATC CTC    360

T   N   K   A   P   E   L   L   K   D   L   D   T   F   L   G   D   K   K   W      140
ACC AAC AAG GCC CCC GAG CTG CTA AAA GAT TTG GAT ACT TTC TTA GGG GAC AAG AAG TGG    420

F   V   G   K   S   V   T   W   A   D   F   Y   W   D   V   C   S   T   T   L      160
TTT GTA GGG AAA TCT GTA ACA TGG GCT GAT TTC TAC TGG GAT GTG TGC AGC ACA ACG CTC    480

L   S   Y   K   A   D   L   A   D   K   Y   P   R   L   L   A   L   R   D   R      180
CTG TCC TAC AAA GCT GAC CTG GCA GAC AAG TAC CCC AGG CTG TTG GCT CTC AGG GAC AGA    540

V   E   A   L   P   A   I   A   A   W   I   Q   K   R   P   K   T   A   I   *      199
GTG GAA GCA CTG CCC GCT ATT GCA GCC TGG ATC CAG AAA AGA CCA AAG ACA GCA ATT TAG    600

actggctgttcagtggaaaaacaaatgcatgtattttggttttcaaacattacaagtgtgctaagttattaattttagaa   680

tcatttaaattaatttcaggtggatatgaagtaccaaatcagaatctagtagagtagagcctgggtagaggtgccattcc   760

aactgcattcccttcatcgctaataaagtgtttggaaaacacggttaatgttctgtgggttcgcagagggagaaaaaata   840

ataaatattactgggcagatataaaaaaaaaa                                                   880
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Figure 1 Nucleotide sequence and primary structure of a cDNA for chicken PGDS

The sequence of the cDNA clone encoding PGDS was determined with the Promega Cycle Sequencing System. The deduced amino acid sequence is shown in single-letter format above that of the cDNA. Position +1 is the first base in the initiation codon; the 36 bases 5′ to the ATG are designated −36 to −1. Numbering of the amino acids includes the initiator methionine residue.

Table 1 Relationship between the translated product of chicken cDNA clone pat.pk0008.b4 and other cloned proteins

The extent of sequence identity and similarity between the chicken GSH-dependent PGDS and other proteins in the SwissProt and GenBank databases was determined with the PILEUP program in GCG. The sequence for the putative GST from *Musca domestica* (housefly) has been entered in GenBank (accession number G409182) but has not yet been published. The sequence for the *Schistosoma japonicum* GST that was published in [25] has been extended and entered in EMBL (accession number Q26513). The sequence for the putative GST from *Caenorhabditis elegans* has been entered in GenBank (accession number U41016). Abbreviation: n.d., not determined.

Species and protein	Reference	Relationship to protein product of pat.pk0008.b4		Catalytic activity	
		Identity (%)	Similarity (%)	GST	PGDS
Rat PGDS (class Sigma GST)	[13]	69.8	84.9	Yes	Yes
Ascaris suum, GST1	[49]	43.2	62.8	Yes	No*
Squid S-crystallin, SL11	[22]	41.1	60.4	Yes	n.d.
Musca domestica, GTS-MUSDO	G409182	40.7	63.3	n.d.	n.d.
Onchocerca volvulus, OvGSTA	[50]	37.8	56.6	Yes	No*
Squid hepatopancreas, 1GSQ	[24]	36.7	56.8	Yes	n.d.
Caenorhabditis elegans, R11G1.3	U41016	36.2	57.7	n.d.	n.d.
Schistosoma japonicum, Sj28	[25]	35.9	57.1	Yes	Low*
Chicken CL2 (class Mu GST)	[38]	31.3	53.6	Yes	n.d.
Chicken GTA1 (class Alpha GST)	[39]	30.1	51.3	Yes	n.d.
Chicken CL1 (class Theta GST)	[40]	25.3	51.6	Yes	n.d.

* D. J. Meyer, unpublished work.

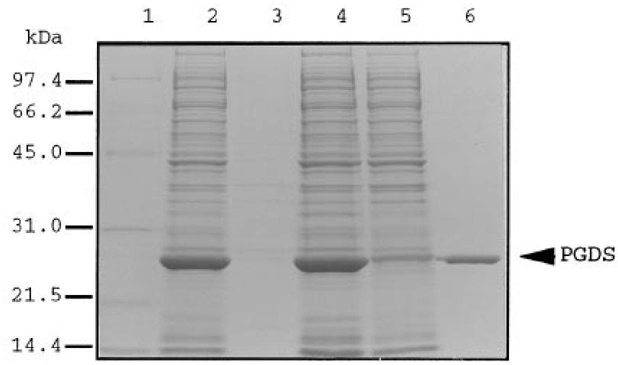

Figure 3 Purification of recombinant chicken PGDS from E. coli

The heterologous expression of chicken PGDS from pET17b in BL21pLysS was examined by SDS/PAGE as described in the Materials and methods section. Purification of PGDS was achieved by affinity chromatography on a column of glutathione–agarose. The samples were loaded as follows: lane 1, molecular mass markers (molecular masses indicated at the left); lane 2, whole bacterial extracts transformed with pET17b containing the ORF of PGDS; lane 3, insoluble pellet from bacterial lysate; lane 4, soluble cell extract; lane 5, material that did not bind to the glutathione–agarose column; lane 6, purified bacterially expressed chicken PGDS.

downstream from the putative TAG termination codon to the poly(A) tail, which comprises at least 50 adenine nucleotides.

As Table 1 shows, the chicken protein encoded by pat.pk0008.b4 shares between 25% and 32% identity with

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                 ++ ***= **                            +   +     +++             ++
chicken PGDS   MPNYKLTYFNLRGRAEICRYLFAYAGIKYEDHRLEGADWPKIKPTIPFGKVPILEV----D-GVIIHQSLAIARYL    71
rat PGDS       ......L...M......I..I...LD.......I.Q.........L....I.V...----E-.LTL.........    71
A.suum         ..Q......DI..LG.GA.LI.HQ..V.F..N..KRE...AL..KT...QL.L...----.-.EVLA..A..Y...    71
squid SL11     ..S.T.Y...G........M...V.SVQ.Q.K.I.L.E.TQF.TKM.CHML....I----.TETQVP..M.IS...    72
housefly*      KNT.T.F...VKAL..PL......G..E...V.VTRDE..AL...M.M.QM.V...----N-.KRV...ISM..F.   109
O.volvulus     .EK.T.....G.....VI.L...L.NVS...N.ITRDE.KYL..RT...H..M.N.----S-.NVLGE.H..ELL.    71
squid Hp GST   ..K.T.H..P.M....L..FVL.AH.EEFT.RVV.M....NL.A.MYSNAM.V.DI----.-.TKMS..MC...H.    71
C.elegans       ME...Q...MM....PA.LI.....VQ.T.E.V.KSA..E..A.T.H..L.V.Y.----.-.AKLA..RV.E...    70
Sj28         MACGHV..I...G.....PI.MILVA..VEF..E.I.FQ..........G.RL..VKITDKRGDVKTMSE......FI    78
                 β1           α1          β2        α2            β3         β4    α3

                                *+ **          *
chicken PGDS   ARESGLAGQTPVEQALADAIVDTIDDFMMLFP-----WAEKNQDVKEKAFNDILTNKAPELLKDLDTFLG----DK   138
rat PGDS       TKNTD...K.EL..CQV..V...L....S...-----...E...L..RT...L..RQ..H.......Y..----..   138
A.suum         G.QF....K..M.E.QV.S.F.QFK...AELRPCFRVL.GFEEGD...VLKEVAVPARDKH.PL.EK..AKS--GS   145
squid SL11     ...F.FY.KNNMDMFKV.CLC.SLFELFNDYMAVYN----EKDAA.KTELQKRFQ.TCLRV.PYMEKT.EANKGGA   144
housefly       .KTV..C.A..W.DLQV.IV....N..RLKIAV---VSY.PEDEI...KLVTLNNEVI.FY.EK.EQTVKDN--.G   180
O.volvulus     GGRF..L.TNDW.E.KIM.V.LN..ELFQKLIPWTH---...TTK.AEL.RNLSESDVMPF.GRYEK..KES--TT   142
squid Hp GST   ...F..D.K.SL.KYRV.E.TE.LQ.I.NDVVKIKF----APEAA..A-VQQNYEKSCKR.APF.EGL.VSNGGGD   140
C.elegans      GKVF....E.DW.T.KM.EL.ACVE..LIEIQPWFK---.QDNAK.VEI.KKLIESTIIPFISAFENI.VTN--GT   141
Sj28           ..KHNMM.D.DD.YYIIEKMIGQVE.VESEYHKTLM----.PPEE...ISKE..NGKV.I..QAICET.KES--TG   148
                           α4                             α5

                        *  **  *                                            *
chicken PGDS   KWFVGKSVTWADFYWDVCSTTL-LSYKADLADKYPRLLALRDRVEAL-PAIAAWIQKRPKTAI       199
rat PGDS       E..I.NY.........I.....-.VL.P..LGI....VS..NK.Q.I-...S...L...Q.KL       199
A.suum         EYM.........LVITDSLASW-E.LIP.FLSGHLQ.KKYIEH.RE.-.N.KK..AE....PY       206
squid SL11     G..I.DQILLC.MMTHAALEN-PIQEN.N.LKE..K.A...T..A.H-.K...Y.K..NN..F       205
housefly       HLALN.-L....V.FAGILDYMNYMV.R.ILEQ..A.RGVV.S.N..-EP.K...E...Q.EV       241
O.volvulus     GHI..NK.SV..LTVFNMLM..DDEV---KLEE..Q.ASFVNKIGQM-.G.KE..K.....YF       201
squid Hp GST   GF...N.M.L..LHCY.ALEV-P.KHTPE.LKDC.KIV...K..AEC-.K...YLK...VRDF       203
C.elegans      GY...DKISY..LAIFHIFWFMNSKILPGALR...K.QEFIEKIT.I-DS.KS..NS....EA       203
Sj28           NLT..DK..L..VVLIASIDHITDLD.EF.TG...EIHKH.KHLL.TS.KL.KYLSE.HA..F       211
                         α6                α7       α8
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Figure 2 Alignment of primary sequences of chicken and rat PGDS with other class Sigma GST proteins

Proteins that possess sequence similarity to chicken PGDS have been aligned with the aid of the Wisconsin software package PILEUP. The amino acid sequences of these proteins were obtained from the SwissProt and GenBank databases, and original references are listed in Table 1. Numbering of the amino acids includes the initiator methionine residue. The complete deduced primary structure of chicken PGDS is shown; the amino acid residues in other proteins that are identical with those in the chicken enzyme are represented by a dot. Spaces inserted to maximize similarity between the proteins are denoted by hyphens. The *Musca domestica* (housefly) sequence contains a 38-residue N-terminal extension that has been omitted from the alignment; the first residue of this protein to be represented is therefore Lys^{39}. The symbols at the top of the alignment indicate those residues in rat PGDS that form the GSH-binding site (+), the PGH_2-binding site (*) and the single residue that interacts with both GSH and PGH_2 (=). The residues in rat PGDS that comprise elements of secondary structure (on the basis of crystallography studies [13,48]) are indicated by solid lines at the bottom of the alignment; α indicates an α-helix; β indicates a β-sheet.

previously cloned classes Alpha, Mu and Theta GSTs that have been isolated from this species [38–40]. However, the protein product of pat.pk0008.b4 shares 97% identity with the N-terminal portion of the chick GST CL5 subunit described by Chang et al. [27]; automated Edman degradation of CL5-5 over 28 cycles yielded an amino acid sequence differing in only one position (Cys^{18} compared with Ser^{18}) from that of the protein encoded by pat.pk0008.b4. It therefore seems likely that the clone from chicken splenic cells represents either the cDNA for CL5 or a closely related GST that is a member of the same family.

Comparison of the chicken protein with proteins from other species demonstrates 70% sequence identity with the rat PGDS subunit (Table 1), and more than 40% identity with proteins from *Ascaris suum* and the squid that have been classified as class Sigma GST. These results indicate that the chicken protein is also a member of the Sigma family. Fourteen of the residues in the predicted chicken protein (Leu^{6}, Tyr^{8}, Phe^{9}, Glu^{16}, Arg^{19}, Asp^{31}, Trp^{39}, Lys^{43}, Pro^{52}, Ser^{64}, Gly^{79}, Lys^{112}, Asp^{150} and Arg^{194}) were found to be invarient among the class Sigma GST, and many of these are predicted from the crystal structure of rat PGDS to form the GSH- and hydrophobic ligand-binding sites (Figure 2). A number of other residues in the chicken protein (Tyr^{4}, Asn^{10}, Gly^{13}, Arg^{14}, Ala^{15}, Phe^{22}, Arg^{33}, Pro^{40}, Pro^{47}, Leu^{54}, Gly^{58}, Gln^{63}, Ile^{67}, Arg^{69}, Leu^{71}, Gly^{76}, Leu^{77}, Thr^{81}, Glu^{84}, Asp^{89}, Asp^{97}, Leu^{128}, Leu^{135}, Gly^{143}, Ala^{149}, Tyr^{171}, Pro^{172}, Leu^{174}, Pro^{185}, Ile^{187}, Ile^{191}, Pro^{195} and Thr^{197}) also seem to be highly conserved among members of this family as they are found in at least seven of the nine structures shown aligned in Figure 2.

The ORF of the chicken cDNA is the same size as that for the rat GSH-requiring PGDS subunit. Although these cDNA species encode proteins with an overall identity of 70%, those regions of secondary structure corresponding to the α2, α3 and α6 of rat PGDS exhibit the greatest conservation (Figure 2). The residues responsible for binding GSH (residues 8, 9, 14, 39, 43, 50, 51, 52, 63, 64 and 97) are all highly conserved between the chicken and rat proteins; indeed, among these residues only a single conservative change exists at residue 51. This sequence information suggests that the chicken protein might function in a glutathione-dependent fashion. From crystallographic studies it has been proposed that 15 residues in rat PGDS are involved in binding PG [13]. Of these, 11 (Arg^{12}, Gly^{13}, Arg^{14}, Glu^{16}, Ile^{17}, Asp^{96}, Met^{99}, Trp^{104}, Tyr^{152}, Cys^{156} and Thr^{159}) are also found in the chicken protein. Three of the four remaining residues in the rat (Met^{11}, Ile^{155} and Leu^{199}) are represented by conservative changes in the chicken (Leu^{11}, Val^{155} and Ile^{199}). The single non-conservative replacement is Met^{100} to Ser^{100}. The positions of these active site residues are shown in Figure 2. The fact that comparisons between the chicken and rat proteins reveal both the GSH-binding and PG-binding residues to be highly conserved suggests that pat.pk0008.b4 encodes a protein that can serve not only as a GST but also as a PG synthase.

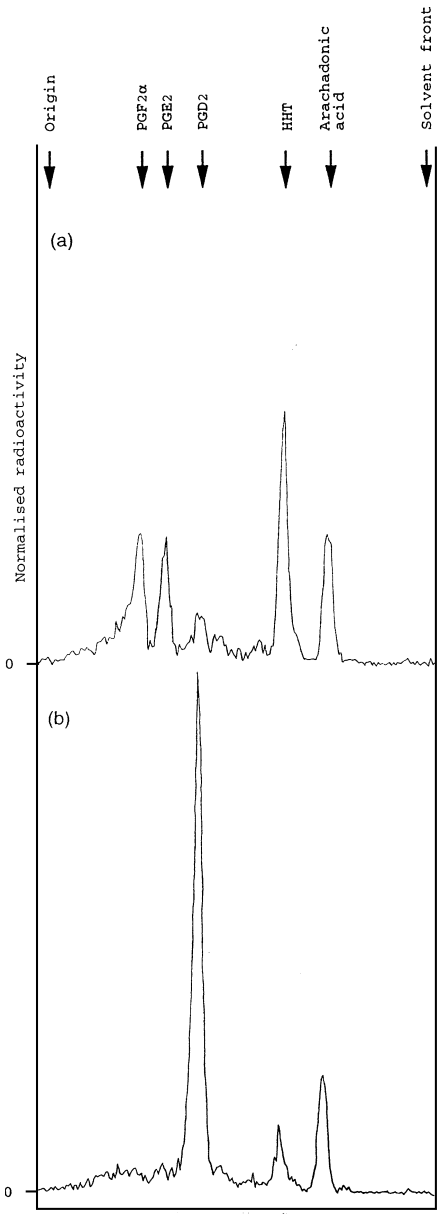

Figure 4 Generation of PGD_2 by recombinant chicken PGDS

PG synthesis from [1-^{14}C]arachidonate was performed with purified COX in a physiological buffer at 37 °C for 1.6 min in the absence (**a**) or the presence (**b**) of 1.3 μg/ml purified recombinant chicken PGDS.

Evidence that the chicken PGDS cDNA encodes a functional PGH2:PGD2 isomerase

To determine the catalytic properties of the protein encoded by pat.pk0008.b4, the ORF was amplified by PCR, ligated into the pET17b expression vector and used to transform *E. coli* as described in the Materials and methods section. The resulting bacterially expressed protein was found to bind the glutathione–agarose affinity matrix, consistent with the notion that it might serve as a GSH-dependent PGDS. Electrophoretic examination of the material that bound to the affinity column showed that it was homogeneous and had an estimated molecular mass of 25 kDa (Figure 3). The yield of affinity-purified protein from 1 litre of bacterial culture was approx. 50 mg.

The possibility that the chicken protein possesses PGH_2:PGD_2 isomerase activity was examined with the coupled assay of Meyer and Thomas [15]. Analysis of the reaction products by TLC showed that the chicken protein specifically converts PGH_2 to PGD_2; it is therefore designated cPGDS (Figure 4). Significantly, the assay also demonstrated that cPGDS did not catalyse the formation of either PGE_2 or $PGF_{2\alpha}$ from PGH_2. The fact that cPGDS has PGD_2 isomerase activity would indicate that it is the chicken orthologue of the rat GSH-requiring PGDS.

Rat PGDS has been shown to catalyse the conjugation of GSH with CDNB [18]; the ability of the chicken orthologue to

Table 2 GST activities of the chicken and rat PGDS

Results are means ± S.D. for four determinations. Data for chick GST CL5-5 are taken from [27] and were performed at 25 °C; data for rat PGDS are from [18] and were performed at 25 °C. A dash indicates that the substrate was not examined.

Substrate	Enzyme ...	Specific activity (μmol/min per mg of protein) cPGDS	Chick CL5-5	Rat PGDS
Aryl halides				
1-Chloro-2,4-dinitrobenzene		97 ± 2	21	3.0
1-Fluoro-2,4-dinitrobenzene		410 ± 14	–	–
1-Iodo-2,4-dinitrobenzene		116 ± 5	–	–
1-Bromo-2,4-dinitrobenzene		128 ± 6	–	–
7-Chloro-4-nitrobenz-2-oxa-1,3-diazole		67 ± 8	–	–
4-Nitrobenzyl chloride		1.4 ± 0.2	–	0†
1,2-Dichloro-4-nitrobenzene		0.053 ± 0.003	–	0†
α,β-Unsaturated carbonyls				
Ethacrynic acid		0.040 ± 0.002	0.6	0†
4-Hydroxynon-2-enal		2.80 ± 0.13	–	–
trans,trans-Deca-2,4-dienal		0.060 ± 0.004	–	–
trans-Non-2-enal		0.019 ± 0.001	–	–
trans-4-Phenylbut-3-en-2-one		0*	–	0†
Organic hydroperoxides				
Cumene hydroperoxide		0.51 ± 0.025	0.72	–
t-Butyl hydroperoxide		0.061 ± 0.002	–	–
Organic isothiocyanates				
Allyl isothiocyanate		12.6 ± 1.4	–	–
Benzyl isothiocyanate		17.6 ± 1.3	–	–
Miscellaneous				
1,2-Epoxy-3-(4′-nitrophenoxy)propane		0*	1.6	0†
Menaphthyl sulphate		0*	–	–
Δ^5-Androstene-3,17-dione		0.021 ± 0.002	0.29	–
4-Nitrophenyl acetate		0.11 ± 0.002	–	–

* No significant activity was obtained in a standard assay with 25 μg of purified PGDS.
† No significant activity was obtained in a standard assay with 2 μg of purified PGDS.

catalyse this reaction was therefore explored. Heterologously expressed cPGDS exhibited transferase activity with CDNB and possessed apparently 30-fold greater activity than the rat enzyme. Chicken PGDS also had high transferase activity towards a range of model aryl halide substrates including 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, 1-bromo-2,4-dinitrobenzene, 1-fluoro-2,4-dinitrobenzene and 1-iodo-2,4-dinitrobenzene (Table 2). The enzyme also displayed a reasonable level of activity with allyl isothiocyanate and benzyl isothiocyanate. Purified cPGDS can catalyse the conjugation of GSH with several α,β-unsaturated carbonyls, although the activity with these compounds is not particularly remarkable. In addition, cPGDS exhibits glutathione peroxidase activity and catalyses the reduction of cumene hydroperoxide.

Inhibition of chicken PGDS

Inhibition of GST activity was studied with CDNB and GSH as substrates. Table 3 shows the IC_{50} results for chicken PGDS with a variety of inhibitors. Cibacron Blue showed the most potent inhibition, whereas the organotin compounds and haematin also demonstrated significant levels of inhibition. *S*-Hexylglutathione did not show a marked inhibition of the activity of cPGDS when compared with some of the class Alpha, Mu and Pi GSTs [41]. Indomethacin, an inhibitor of both COX-1 and COX-2 enzymes, did not show significant inhibition of cPGDS activity.

Table 3 Inhibitors of the transferase activity of chicken PGDS

The ability of non-substrate ligands to inhibit the CDNB–GSH-conjugating activity of heterologously expressed chicken PGDS was measured under standard assay conditions. IC_{50} values were determined on two separate occasions.

Inhibitor	IC_{50} (μM)
Cibacron Blue	0.03
Haematin	0.80
Indomethacin	300
S-Hexylglutathione	100
Sulphasalazine	> 100
Tributyltin acetate	0.90
Triphenyltin chloride	0.10
Triethyltin bromide	0.30

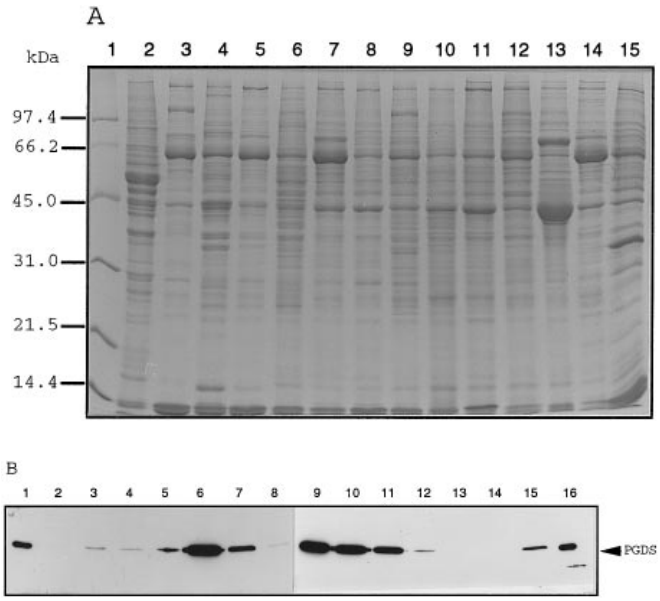

Figure 5 Tissue-specific expression of PGDS in the chicken

Extracts from chicken organs were subjected to SDS/PAGE in 12% (w/v) polyacrylamide resolving gels. (**A**) Electrophoretically resolved chicken proteins stained with Coomassie R250; (**B**) immunoblotting of the same samples with antibody raised against the rat PGDS and development with enhanced chemiluminescence as described in the Materials and methods section. In (**A**) and (**B**) the gel was loaded with either 0.02 μg of purified protein or 10 μg of whole tissue protein as follows: lane 1, molecular mass markers (**A**; molecular masses indicated at the left) or recombinant chicken PGDS (**B**); lane 2, brain; lane 3, thymus; lane 4, heart; lane 5, lung; lane 6, liver; lane 7, pancreas; lane 8, spleen; lane 9, kidney; lane 10, small intestine; lane 11, colon; lane 12, ovary; lane 13, oviduct; lane 14, skin; lane 15, bone marrow; lane 16 in (**B**), recombinant chicken PGDS. Two separate gels were loaded in (**B**): lanes 1–8 and lanes 9–16.

Tissue distribution of PGDS

Antisera against the rat GSH-dependent PGDS were found to cross-react with chicken PGDS. These antibodies were therefore used to probe various avian tissues. In Western blotting experiments a single immunoreactive band was detected in cytosolic extracts prepared from several chicken tissues that co-migrated with the heterologously expressed cPGDS (Figure 5). The PGDS

was found in highest amounts in chicken liver, kidney, small intestine and colon. It was expressed in moderate amounts in pancreas, bone marrow, lung and ovary. Low levels of cPGDS were also detected in spleen, thymus, heart and brain, but it was not observed in oviduct or skin. Therefore the structure and function, but not the tissue-specific expression, seem to be conserved between chicken and rat PGDS.

DISCUSSION

Distribution of PGDS in Nature

This paper describes a previously unrecognized PG synthase that was cloned as an EST from chicken splenic T cells. Heterologous expression of the chicken cDNA showed that it encodes a protein that specifically catalyses the isomerization of PGH_2 to PGD_2; it is therefore referred to as cPGDS. So far, the chicken cDNA is only the second clone to have been isolated that has been shown to encode a functional GSH-dependent PGDS. The first such cDNA clone to be isolated was that for the rat haemopoietic PGDS, and comparison between the primary structures of the chicken and rat PGDS proteins shows that they share 70% identity and 85% similarity.

In addition to the chicken and rat, evidence suggests that mouse [42] and human [43] also possess GSH-dependent PGDS proteins. The cDNA species for the mouse and human synthases have not yet been described, and hence their relationship to the chicken and rat enzymes is not known. Nevertheless, antibodies raised against rat PGDS cross-react with a polypeptide in murine mast cells that co-migrates during electrophoresis with the immunogen [42], suggesting that the two rodent synthases are structurally similar. The chicken synthase described in this paper is therefore of particular interest as it is the first example of a non-mammalian GSH-dependent PGDS and might give a valuable insight into structure–function aspects of this group of enzymes.

Sequence alignments place cPGDS in the class Sigma GST family. Although this class of transferase is represented in many species, most of the proteins have been isolated by molecular cloning and little is known about their catalytic properties. Most of the class Sigma GSTs in invertebrates show poor conservation of those active-site residues in α4 and α6 of rPGDS that are implicated in PG binding (see Figure 2). For example, whereas cPGDS shares 11 of the 15 residues that form the PGH_2-binding site in rPGDS, fewer than half of these residues are conserved in the other class Sigma GSTs shown in Figure 2. Thus only 5 of the 15 residues in rPGDS that comprise the PGH_2-binding site are present in the class Sigma GSTs from squid digestive gland, squid eye and *Onchocerca volvulus*. Only 4 of these 15 residues are found in the class Sigma GSTs from *A. suum* and *Schistosoma japonicum*, and merely 3 of the 15 residues are found in the housefly and *C. elegans* proteins. It therefore seems unlikely that all transferases in the Sigma class are able to catalyse the isomerization of PGH_2 to PGD_2. Indeed, this supposition has been found to be correct as the *A. suum* and *O. volvulus* GSTs lack PGDS activity (D. J. Meyer, unpublished work). Thus only a subfamily of class Sigma GSTs exhibit PGDS activity. It remains to be established how many other class Sigma GSTs from other non-mammalian species besides the chicken exhibit PGDS activity.

Biochemical activities of chicken PGDS

The transferase activity of cPGDS has been investigated, in part because of the absence of data about the catalytic properties of class Sigma GSTs. The present study shows that the chicken enzyme not only metabolizes PGH_2 but also has a broad specificity towards many model GST substrates. It exhibits particularly high activity towards certain aryl halides, but this activity is selective in that a number of model substrates that can be included in this category do not serve as substrates for the enzyme. For example, cPGDS was found to be active with a series of halide-substituted 2,4-dinitrobenzenes and with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole. It had only low activity towards 4-nitrobenzyl chloride and was essentially inactive with 1,2-dichloro-4-nitrobenzene. The enzyme had good activity towards allyl isothiocyanate and benzyl isothiocyanate, compounds that are abundant in many edible plants including cauliflower, broccoli and cabbage [44]. Chicken PGDS has a small amount of transferase activity with α,β-unsaturated carbonyls; among such compounds it was found to have the highest activity with 4-hydroxynon-2-enal, a major product of lipid peroxidation [31]. The chicken enzyme also shows modest peroxidase activity towards cumene hydroperoxide. The substrate specificity of the chicken class Sigma GST towards aryl halides and α,β-unsaturated carbonyls distinguishes it from class Alpha, Mu, Pi, Theta and Zeta GSTs [16,45].

Determination of the substrate specificity of cPGDS allows comparison with the activity of GST CL5-5 purified from chicken liver [27]. As Table 2 shows, significant differences exist between the 3-ketosteroid isomerase activity and ethacrynic acid–GSH-conjugating activity of the two enzymes. These differences in catalytic activity indicate that cPGDS and CL5-5 are possibly separate proteins, but this interpretation assumes that the protein is not subject to post-translational modification. It is also possible that the differences in the activity of cPGDS and CL5-5 might simply be due to variations in the assay conditions used in the present work and those of Chang et al. [27]. In the context of functional similarity between cPGDS and CL5-5, it is interesting that neither protein seems to have a high affinity for *S*-hexylglutathione, as this conjugate is a weak inhibitor of cPGDS (Table 3) and the CL5-5 protein failed to bind to an *S*-hexylglutathione-agarose affinity column [27]. The question of identity between cPGDS and GST CL5-5 will require further study before it is resolved, but they are clearly closely related because in their 28 N-terminal amino acid residues only one difference between them has been observed. The fact that this single difference involves the replacement of Cys^{18} in cPGDS by serine in the GST CL5-5 protein might not be genuine because cysteine can be difficult to identify by amino acid sequencing unless it is modified before analysis; unfortunately, it is unclear whether CL5 was derivatized to allow the recovery of Cys during sequencing [27].

Although rat PGDS was first purified from spleen many years ago, a paucity of material has precluded extensive characterization of the enzyme. Urade et al. [18] showed that rPGDS was active with CDNB but these workers were unable to demonstrate activity with other transferase substrates such as 1,2-dichloro-4-nitrobenzene, 4-nitrobenzyl chloride, ethacrynic acid, *trans*-4-phenylbut-3-en-2-one and 1,2-epoxy-3-(4′-nitrophenoxy)propane. By using a recombinant DNA approach to enable the isolation of large amounts of cPGDS, a sufficient amount of protein has been generated in the present study to allow a study of the GST activity of the chicken protein with a broad range of substrates. Although cPGDS was shown to be active with most of the model substrates examined, it was found to demonstrate low activity with those compounds that were not metabolized by rPGDS. It is therefore expected that the chicken and rat enzymes display similar GST activities; this prediction must be examined formally.

The transferase activity of cPGDS has been found to be inhibited by a range of non-substrate ligands that serve as inhibitors for other classes of GST (Table 3). Although this finding is not particularly surprising, as Urade et al. [18] noted the synthase activity of rPGDS is inhibited by Indocyanine Green (IC_{50} 2μM), bilirubin (IC_{50} 150μM) and CDNB (IC_{50} 5000μM), the present study has revealed that Cibacron Blue and triphenyltin chloride are particularly potent inhibitors, having IC_{50} values at least one order of magnitude lower than those reported previously for Indocyanine Green. It is predicted from these results that the activity of PGDS in the human will be inhibited by xenobiotics and therefore the enzyme could be targeted therapeutically, for example in patients with mastocytosis that synthesize inappropriate amounts of PGD_2 [46].

Expression of PGDS in the chicken

Western blotting experiments have revealed that cPGDS is subject to tissue-specific expression. The chicken organs that contain the highest levels of PGDS are liver, kidney, small intestine and colon, with only small amounts of immunoreactive protein being found in the spleen. In contrast, rat spleen contains large amounts of PGDS (it represents approx. 0.1% of cytosolic protein [18]), whereas rat liver, kidney, small intestine and colon contain small but detectable amounts of the isomerase. The immunoblotting experiments described in this study showed PGDS to be undetectable in chicken skin, although previous work has shown that in the rat PGDS is present in substantial amounts in skin, being located in Langerhans cells, histiocytes and mast cells [47]. In addition, PGDS was not found in chicken oviduct, whereas it is abundant in this organ in the rat [13]. These differences in the tissue-specific expression of PGDS in chicken and rat are surprising and suggest that the physiological roles of the two synthases might differ in these two species. The high levels of PGDS in chicken liver, kidney and the gastrointestinal tract suggest that it serves a detoxification role as well as a role in PG biosynthesis. Such a dual function for the rat enzyme is implausible because its expression in rat tissues is more restricted than that of the chicken enzyme. The rat enzyme is located in antigen-presenting cells, particularly histiocytes, dendritic cells and Kupffer cells of various tissues, rather than cells involved in detoxification, and it is postulated that it has a role in dictating the progress of immune responses [19]. Further studies are required to determine whether during the process of evolution the PGDS subfamily of class Sigma GSTs has acquired a specific role in PG synthesis at the expense of a more general detoxification function. Should this hypothesis be correct, the chicken might reflect an earlier, less specialized, functional stage in the evolutionary history of this family of enzymes.

Concluding remarks

During the present study a cDNA has been characterized that encodes a chicken GSH-dependent PGDS. The availability of this clone will allow transfection studies to be undertaken that are aimed at establishing the physiological effects of PGD_2 and the J_2 series of prostanoids.

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