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REVIEW ARTICLE

Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily

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The glutathione transferases (GSTs; also known as glutathione S-transferases) are major phase II detoxification enzymes found mainly in the cytosol. In addition to their role in catalysing the conjugation of electrophilic substrates to glutathione (GSH), these enzymes also carry out a range of other functions. They have peroxidase and isomerase activities, they can inhibit the Jun N-terminal kinase (thus protecting cells against H_2O_2-induced cell death), and they are able to bind non-catalytically a wide range of endogenous and exogenous ligands. Cytosolic GSTs of mammals have been particularly well characterized, and were originally classified into Alpha, Mu, Pi and Theta classes on the basis of a combination of criteria such as substrate/inhibitor specificity, primary and tertiary structure similarities and immunological identity. Non-mammalian GSTs have been much less well characterized, but have provided a disproportionately large number of three-dimensional structures, thus extending our structure–function knowledge of the superfamily as a whole. Moreover, several novel classes identified in non-mammalian species have been subsequently identified in mammals, sometimes carrying out functions not previously associated with GSTs. These studies have revealed that the GSTs comprise a widespread and highly versatile superfamily which show similarities to non-GST stress-related proteins. Independent classification systems have arisen for groups of organisms such as plants and insects. This review surveys the classification of GSTs in non-mammalian sources, such as bacteria, fungi, plants, insects and helminths, and attempts to relate them to the more mainstream classification system for mammalian enzymes. The implications of this classification with regard to the evolution of GSTs are discussed.

Key words: detoxification, GST, polymorphism, protein, xenobiotic.

INTRODUCTION

It is now well established that the glutathione transferases (GSTs; known formerly as glutathione S-transferases; reviewed in [1–7]) play a key role in phase II of enzymic detoxification (Scheme 1). However, recent developments, such as the availability of large-scale genome data, expressed sequence tag (EST) databases [9,10], novel sequence alignment procedures [11] and the determination of three-dimensional structures by X-ray crystallography [2,3], have greatly extended our knowledge of structure–function relationships in this important enzyme superfamily. While mammalian GSTs have been extensively investigated and classified according to generally agreed criteria, a number of novel GST classes were identified first in non-mammalian sources, and only later recognized in mammals. Moreover, a disproportionately large number of crystal structures have been obtained for non-mammalian GSTs. These developments have allowed us to recognize novel functions which, in some cases, were not originally associated with GSTs. These enzymes carry out a wide range of functions in cells, such as the removal of reactive oxygen species and regeneration of S-thiolated proteins (both of which are consequences of oxidative stress), catalysis of conjugations with endogenous ligands, and catalysis of reactions in metabolic pathways not associated with detoxification. The classification system originally developed for mammalian GSTs has proved robust enough to be extended to non-mammalian enzymes; conversely, new classes described originally in non-mammalian sources have later been found also in mammals. This review focuses on the classification of non-mammalian GSTs, with particular emphasis on their significance for extending our knowledge of structure–function relationships in these enzymes and on the implications for the evolution of this complex multifunctional superfamily.

ROLE OF GSTs IN ENZYMIC DETOXIFICATION

Living organisms are continuously exposed to non-nutritional foreign chemical species. These xenobiotics may interact deleteriously with an organism, causing toxic and sometimes carcinogenic effects [12]. While the threat posed by such compounds has increased greatly in the last two centuries due to the range of novel man-made chemicals introduced into the environment in that time, it has probably existed as long as life itself. Naturally occurring toxic compounds include plant and fungal toxins (e.g. plant phenols and aflatoxins) and reactive oxygen species, such as the superoxide radical and hydrogen peroxide (H_2O_2). The ability to survive the threat posed by endogenously produced and/or xenobiotic compounds probably represents a biological adaptation fundamental to survival [4,13].

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; Dnp-SG, dinitrophenol–glutathione conjugate; EST, expressed sequence tag; GST, glutathione transferase; MAPEG, membrane-associated proteins in eicosanoid and glutathione metabolism; MIF, migration inhibitory factor; MOAT, multispecific organic ion transporter; MRP, multidrug-resistance-associated protein; single-letter prefixes denote species of origin (r, rat; m, mouse; h, human).

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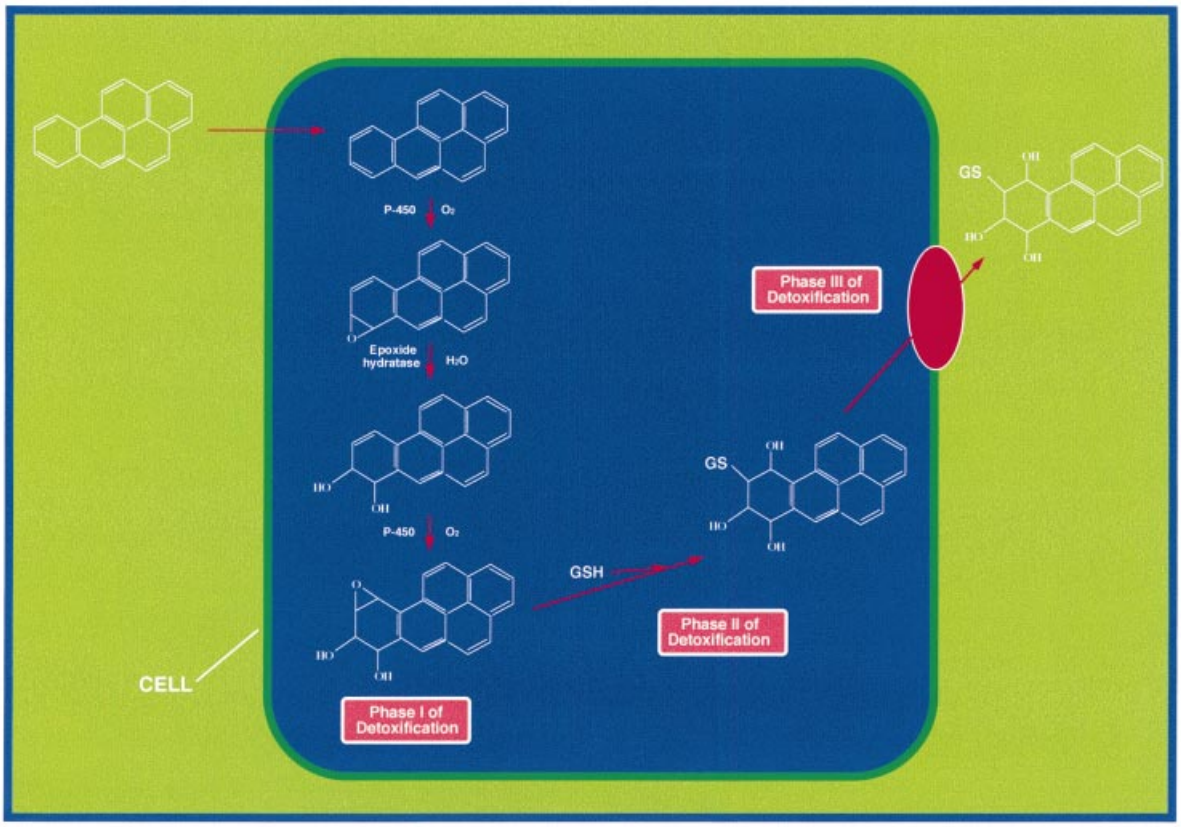

Scheme 1 Overview of enzymic detoxification

A possible metabolic fate of benzo[*a*]pyrene is illustrated, although many other metabolites are also formed. The xenobiotic diffuses freely across the plasma membrane, where it becomes a substrate for the cytochrome P450 system, resulting in the formation of an epoxide. This in turn becomes a substrate for epoxide hydratase. The diol product of this reaction can again be acted upon by cytochrome P450 to form a carcinogenic and mutagenic diol-epoxide derivative of the xenobiotic. Both of these enzymes are microsomal, and form phase I of enzymic detoxification. GSTs are mainly cytosolic phase II enzymes which catalyse conjugation to GSH. The GSH–xenobiotic conjugate is too hydrophilic to diffuse freely from the cell, and must be pumped out actively by a transmembrane ATPase such as the GS-X pump [8]. This results in the unidirectional excretion of the xenobiotic from the cell, since the hydrophilic GSH moiety prevents re-diffusion across the plasma membrane. Ultimately, this conjugate is excreted from mammals as mercapturic acids.

Along with strategies such as sequestration, scavenging and binding, catalytic biotransformation evolved as an important biochemical protection mechanism against toxic chemical species. Cells possess an impressive array of enzymes capable of biotransforming a wide range of different chemical structures and functionalities. The enzymic detoxification of xenobiotics has been classified into three distinct phases which act in a tightly integrated manner. Phases I and II involve the conversion of a lipophilic, non-polar xenobiotic into a more water-soluble and therefore less toxic metabolite, which can then be eliminated more easily from the cell (phase III; Scheme 1). Phase I is catalysed mainly by the cytochrome P450 system. This family of microsomal proteins is responsible for a range of reactions, of which oxidation appears to be the most important [14].

Phase II enzymes catalyse the conjugation of activated xenobiotics to an endogenous water-soluble substrate, such as reduced glutathione (GSH), UDP-glucuronic acid or glycine. Quantitatively, conjugation to GSH, which is catalysed by the GSTs, is the major phase II reaction in many species. GSTs can catalyse nucleophilic aromatic substitutions, Michael additions to α,β-unsaturated ketones and epoxide ring-opening reactions, all of which result in the formation of GSH conjugates [6] and the reduction of hydroperoxides, resulting in the formation of oxidized glutathione (GSSG) [6,13]. Several transport mechanisms exist for the elimination of glutathione conjugates, including an ATP-dependent GS-X pump [8], a multispecific organic anion transporter (MOAT) [15], a broad-specificity anion transporter of dinitrophenol S–GSH conjugates (Dnp-SG ATPase) [16], P-glycoprotein (the 170 kDa multidrug resistance pump) [17] and the multidrug-resistance-associated protein (MRP; a 190 kDa glycoprotein) [18,19]. While some functional relationships exist among the GS-X pump, MOAT, Dnp-SG ATPase and MRP, the structural relationship of these proteins with the P-glycoprotein remains unclear [4,20].

GSTs are dimeric, mainly cytosolic, enzymes that have extensive ligand binding properties in addition to their catalytic role in detoxification [21–23]. They have also been implicated in a variety of resistance phenomena involving cancer chemotherapy agents [24,25], insecticides [26,27], herbicides [7,28] and microbial antibiotics [29]. A separate microsomal class of GSTs exists which is quite distinct from the cytosolic enzymes, and is designated as 'membrane-associated proteins in eicosanoid and glutathione' metabolism (MAPEG) [30].

The GSTs comprise a complex and widespread enzyme superfamily that has been subdivided further into an ever-increasing number of classes based on a variety of criteria, including amino acid/nucleotide sequence, and immunological, kinetic and tertiary/quaternary structural properties. Representative crystal

structures are available for most classes and, despite limited overall sequence identity, these follow generally similar folds, with structural differences concentrated especially around the active site and at the inter-subunit interface. GST genes and proteins from mammalian sources (particularly rat, human and mouse) have been especially well characterized, but studies of GSTs from non-mammalian sources have revealed the existence of several new classes and thus greatly extended our knowledge of the structural and functional diversity of these proteins.

CLASSIFICATION CRITERIA FOR MAMMALIAN GSTs

While there are no clearly established criteria concerning the extent of sequence similarity required for placing a GST in a particular class, it is generally accepted that GSTs share greater than 60 % identity within a class, and those with less than 30 % identity are assigned to separate classes. Much emphasis tends to be placed on the primary structure at the N-terminus because, within the classes, this region tends to be better conserved than others, as it includes an important part of the active site. This region contains a catalytically essential tyrosine, serine or cysteine residue that interacts with the thiol group of GSH, thus lowering its pK_a to a value of approx. 6–7 from its normal value of around 9.0. This is thought to be a key component of catalysis in GSTs [2,31,32].

Within a particular class, clearly defined subfamilies, which represent unique subunit types, can sometimes be identified. Each subfamily may include as many as five separate, highly homogeneous, polypeptides, which may share greater than 90 % identity [4,33]. Amino acid residues at positions 60 and 80 appear to be particularly well conserved [34]. The hypothesis that the classes represent separate families of GSTs is further supported by the distinct structures of their genes and their chromosomal localizations. The Alpha-, Mu-, Pi- and Theta-class GST genes differ markedly from each other in size and in intron/exon structure [4], and there is a trend for human GST genes to be found in class-specific clusters [35].

Hayes and Mantle [36] demonstrated the use of immunoblotting techniques in identifying the tissue-specific expression of GSTs and in the determination of immunological relationships between individual subunits. Polyclonal antisera raised against a particular GST class will often cross-react with the same class from other species. No cross-reactivity is generally noted between GST classes, even within the species from which the original antigen was derived. This points to recognition by antibodies of solvent-accessible topological features that are conserved within classes.

Kinetic properties, such as substrate specificities and inhibitor sensitivities, can also sometimes be used to distinguish different GST isoenzymes. However, due to broad and often overlapping values, they do not give as definitive a distinction between GST classes as sequence or immunological analysis. In comparing mammalian GSTs, kinetic properties are important when a low level of sequence identity is present [37]. A more comprehensive analysis of kinetic properties can be made by multivariate analysis, in which a larger number of variables ($n > 2$) may be represented [34]. Mannervik et al. [37] identified three classes of cytosolic GSTs common to several mammalian species using multivariate analysis. Specific activity values for nine substrates and IC_{50} values for 11 inhibitors were used as variables to characterize 15 GST isoenzymes. They were clustered into three regions, and each region contained the members of a class, as defined by other functional and structural properties. In contrast with mammalian enzymes, however, substrate specificity is a rather poor criterion for extending the mammalian classes into non-mammalian sources, as is clear from studies of plant, microbial and insect GSTs (see below).

Table 1 Some useful classification criteria for GSTs

Criterion	Example	Ref.
Primary structure comparisons	Alpha/Mu/Pi classes	[42]
	Theta class	[43]
	Kappa class	[44]
	Zeta class	[45]
	Omega class	[39]
Immunoblotting	Alpha/Mu	[36]
	MIF	[46]
	Insect classes I and II	[47]
	Fasciola hepatica	[48]
Kinetic properties		
Substrate specificity/affinity	Alpha/Mu/Pi	[37]
	Mu	[33]
	Theta	[49]
Inhibitor sensitivity	Alpha/Mu/Pi	[34,37]
Tertiary structure: active site	Alpha/Mu/Pi	[2,3]
	Theta	[50]
	Omega	[39]
	Beta	[41]
	Sigma	[51,52]
Quaternary structure		
Ability to hybridize into dimers	Mu/Alpha	[38]
Inter-subunit interface	Hydrophobic lock and key in Alpha/Mu/Pi/Theta classes	[2,3]
	Polar interface in Beta class	[41]

GST subunits appear to be only capable of hybridizing with subunits from the same class [4]. Formation of homo- and hetero-dimers presumably allows the formation of a larger number of enzymes from a limited number of genes. For example, a pair of homodimers, when denatured in 8 M urea *in vitro* followed by dialysis, can hybridize together to form three isoenzymes (two homodimers and a heterodimer) composed of one of each of the subunits [38]. A similar process of random dimer formation is thought to happen post-translationally [4]. The availability of representative crystal structures from the main classes has explained these findings by revealing that structural interactions at the inter-subunit interface are crucial for dimer assembly and stability, and are often class-specific (see below). The ability to hybridize together to form a stable GST dimer may therefore be regarded as a criterion of classification, and unusual architecture at the interface between subunits has been used to define novel GST classes (e.g. see [39–41]).

These and other criteria useful for GST classification are summarized in Table 1.

ALPHA/MU/PI CLASSES

Based on a combination of the criteria discussed above, mammalian cytosolic GSTs were classified into the Alpha, Mu and Pi classes [42]. Several enzymes were recognized as belonging to the Alpha and Mu classes, while the Pi class originally contained only one protein, GST P. At least six distinct Mu-class subunits (M1, M2, M3, M4, M5* and M6*, where * denotes that a full primary structure is not available for this GST) have been identified in the rat, with homologous gene loci for the first five of these in humans. Polymorphisms in genes coding for enzymes involved in protection against oxidative stress have been impli-

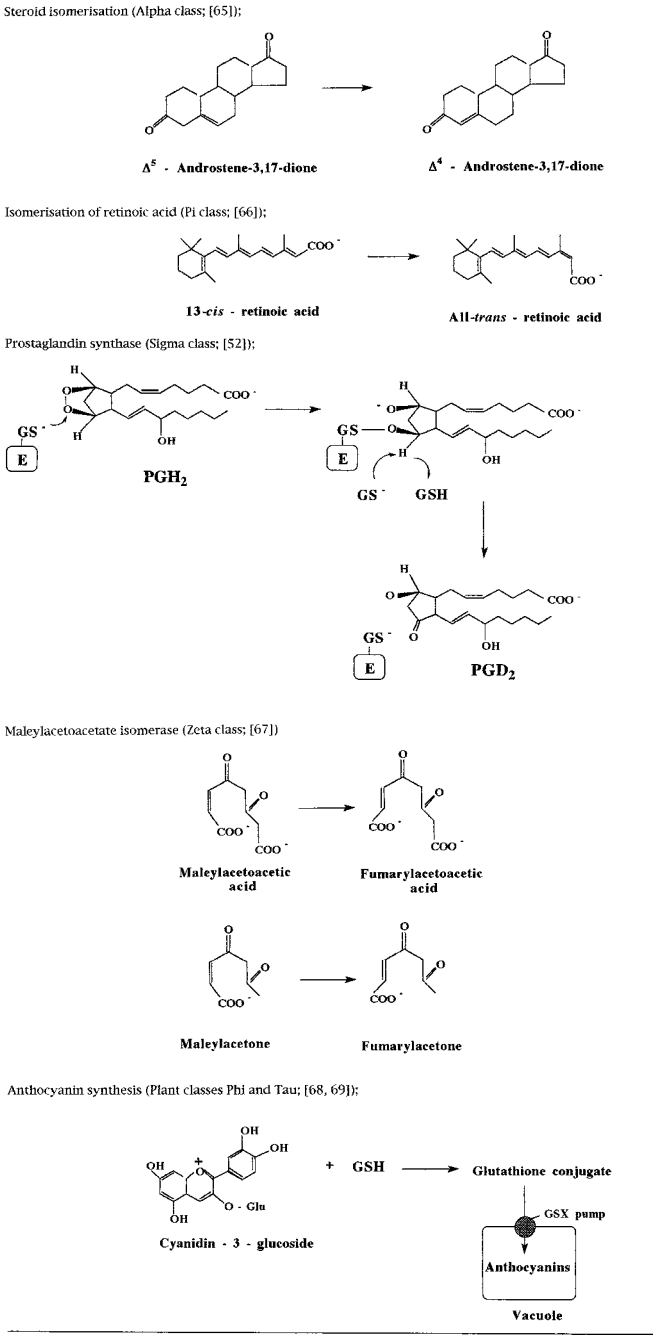

Figure 1 Some reactions catalysed by GSTs in addition to their detoxification function

cated in the predisposition of individuals to disease states such as cancer [53]. In the case of the Mu class, four allelic variants at the GST M1 locus have been identified in the human population [54,55]. Of these four, the null allele is present in 50% of the human population, which may predispose certain individuals to greater risk from toxic xenobiotics [56–58]. It has been proposed that rGSTM5, together with mGST M5 and hGST M3, form a distinct subclass of the Mu class, based on kinetic and subunit hybridization criteria [33,59,60].

A large number of Alpha-class GSTs, comprising at least six types of subunits (A1, A2, A3, A4, A5 and A6*), have been identified in the rat [4,61], with homologous gene loci in humans for A1–A4 [62,63]. Comparison of substrate preferences and sequence similarities has allowed the identification of subgroups within the Alpha class; it was found that the A4 subunit has particularly high activity with ethacrynic acid, lipid hydroperoxides and 4-hydroxyalkenals, while sharing sequence and immunological similarities with the other Alpha-class enzymes [64]. Alpha-class GSTs have also been discovered to possess steroid isomerase activities in rat ovary and testis [65]. This is an example of an endogenous non-detoxification function for Alpha-class GSTs. Various other isomerase activities have been discovered in other GST classes (Figure 1). For example, hGST P1-1 has been shown to catalyse the isomerization of 13-*cis*-retinoic acid to all-*trans*-retinoic acid [66]. Since this isomerization is independent of GSH, it is distinct from most other isomerization reactions reported for GSTs, which usually require GSH. Polymorphisms have been identified in several human phase II enzymes and in GSTs in particular [11,70,71]. In the case of Alpha-class GSTs, a polymorphism was discovered at the A2 locus [62,72]. A number of Pi-class polymorphisms have now also been described ([73–75]; reviewed in [71]). In addition to their isomerization and GSH-conjugation activities, in mammals these enzymes contribute to defence against oxidative stress, by virtue of both their selenium-independent GSH peroxidase activities (Alpha class) [76] and their role as inhibitors of the Jun N-terminal kinase (Pi class), which protects cells against H_2O_2-induced cell death [77,78].

CRYSTAL STRUCTURES OF GSTs

Representative crystal structures are available for all the cytosolic GST classes mentioned in this article, with the exception of the Kappa class (see below). A comparison of some structures is provided in Figure 2. Despite the low overall level of sequence identity across the classes, all the structures follow a similar canonical fold, with each subunit consisting of two distinct domains (Figure 3) [3,80–82]. The N-terminal domain 1 (approx. residues 1–80) adopts a topology similar to that of the thioredoxin fold [3,85,86], consisting of four β-sheets with three flanking α-helices (Figure 4). This fold occurs in several proteins of limited sequence identity from other enzyme families, which appear to have evolved to bind cysteine or GSH. Examples are DsbA (the bacterial enzyme equivalent to protein disulphide isomerase) [87], glutaredoxin [88] and glutathione peroxidase [89]. The fold consists of distinct N-terminal and C-terminal motifs which have a $\beta\alpha\beta$ and $\beta\beta\alpha$ arrangement respectively, and which are linked by an α-helix (α-2 in Figure 4). The former begins with an N-terminal β-strand (β-1), followed by an α-helix (α-1) and then a second β-strand (β-2) which is parallel to β-1. A loop region leads into a second α-helix (α-2), which connects with the C-terminal motif. This motif consists of two sequential β-strands (β-3 and β-4), which are antiparallel and which are followed by a third α-helix (α-3) at the C-terminus of the fold. The four β-sheets are essentially in the same plane, with two helices (α-1 and α-3) below this plane and α-2 above it, facing the solvent. The loop that connects α-2 to β-3 features a characteristic proline residue which is in the less favoured *cis* conformation and is highly conserved in all GSTs. This is referred to as the *cis*-Pro loop which, while playing no direct role in catalysis, appears to be important in maintaining the protein in a catalytically competent structure [90]. In GSTs, domain 1 is highly conserved and provides most of the GSH binding site. It is connected to domain 2 by a short linker sequence, as shown in Figure 3 [2].

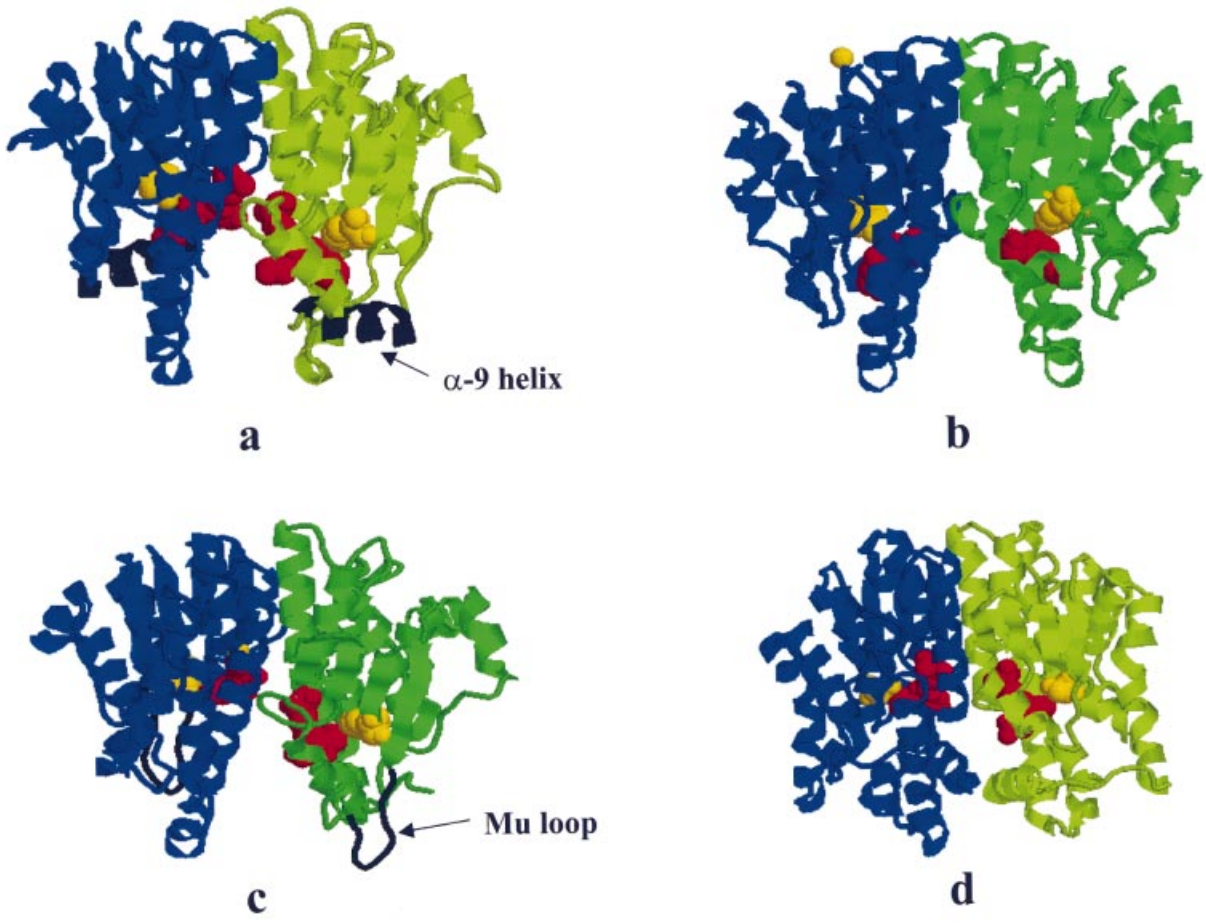

Figure 2 Mammalian GST structures

Structures are visualized using the RasMol program [RasMol v.2.7.1.1. (http://www.bernstein-plus-sons.com/software/rasmol/) modified by Bernstein, H. J. (1998–2001), based on original v.2.6 (1995) and v.2.6.4 (1998) by Sayle, R. and previous modifications by Mueller, A. (1998)]. Subunits are distinguished by colour (green and blue), and structures are represented to emphasize the relative arrangements around the active site of the right-hand subunit and the inter-subunit cleft. Catalytically essential tyrosine or serine residues are represented in space-filling mode and highlighted in yellow, while the ligand with which the enzyme was co-crystallized is shown in red, identifying the location of the active site. Class-specific features of the Alpha and Mu structures are shown in black. Protein database codes are given in parentheses: (**a**) human Alpha class (1GUH; [80]); (**b**) human Pi class (1GSS; [81]); (**c**) rat Mu class (6GST; [82]); (**d**) human Theta class (1LJR; [83]). A multi-media adjunct to this Figure can be viewed at http://www.BiochemJ.org/bj/360/bj3600001add.htm

Domain 2 (approx. residues 87–210) begins at the C-terminus of the linker sequence, and consists of five α-helices in the case of the Pi and Mu classes [51,91] and six α-helices in the case of the Alpha class [80] (the number of helices in domain 2 varies widely between classes, as is described below for each individual case). The C-terminal domain is less similar between the three mammalian classes than the N-terminal domain (Figures 2 and 3) [2,3]. It contributes most of the residues that interact with the hydrophobic second substrate, as well as contributing a highly conserved aspartic acid residue (occurring in helix α-4) to the GSH binding site. Differences in the C-terminal domain may be responsible for differences in substrate specificity between the three classes [3].

Comparison of the mammalian Alpha/Mu/Pi structures reveals several major points of similarity, as well as crucial points of difference [2]. These become especially important when comparing Alpha/Mu/Pi structures on the one hand with those of other classes, which are discussed later in this article. There is clear similarity in overall domain structure, despite differences in the detailed structure of domain 2. There are also striking similarities in the types of interactions occuring at the inter-subunit interface (Figure 2). These are dominated by hydrophobic interactions between residues from domain 1 of one subunit and domain 2 of the other. An aromatic residue (Phe-52 in Alpha, Phe-56 in Mu and Tyr-49 in Pi) acts as a 'key' extending from the loop preceding β-3 which fits into a hydrophobic 'lock' provided by helices α-4 and α-5 of the other subunit. The interface is approx. 25 Å $\times$ 35 Å and, at a height of approx. 25 Å, diverges to create a V-shaped crevice which is solvent-accessible [80] (1Å = 0.1 nm).

A total of 26 residues are conserved between the sequences of the three proteins, which represent 20–32 % pairwise identity [2,80]. All three enzymes bind GSH in an extended conformation and possess an N-terminal tyrosine residue (located in β-1) which is within hydrogen-bonding distance of the thiol group of GSH (Figure 5). Site-directed mutagenesis experiments have shown this residue to be catalytically essential (for reviews, see [1,3]). Moreover, the main-chain φ angle around this residue is close to 180°, which introduces an unusual twist into β-1, presumably facilitating catalysis [86]. A SNAIL/TRAIL motif (i.e. Ser-Asp-Ala-Ile-Leu/Thr-Arg-Ala-Ile-Leu) has been observed in the α-3 helix of domain 1 which is present in most GSTs [92], and

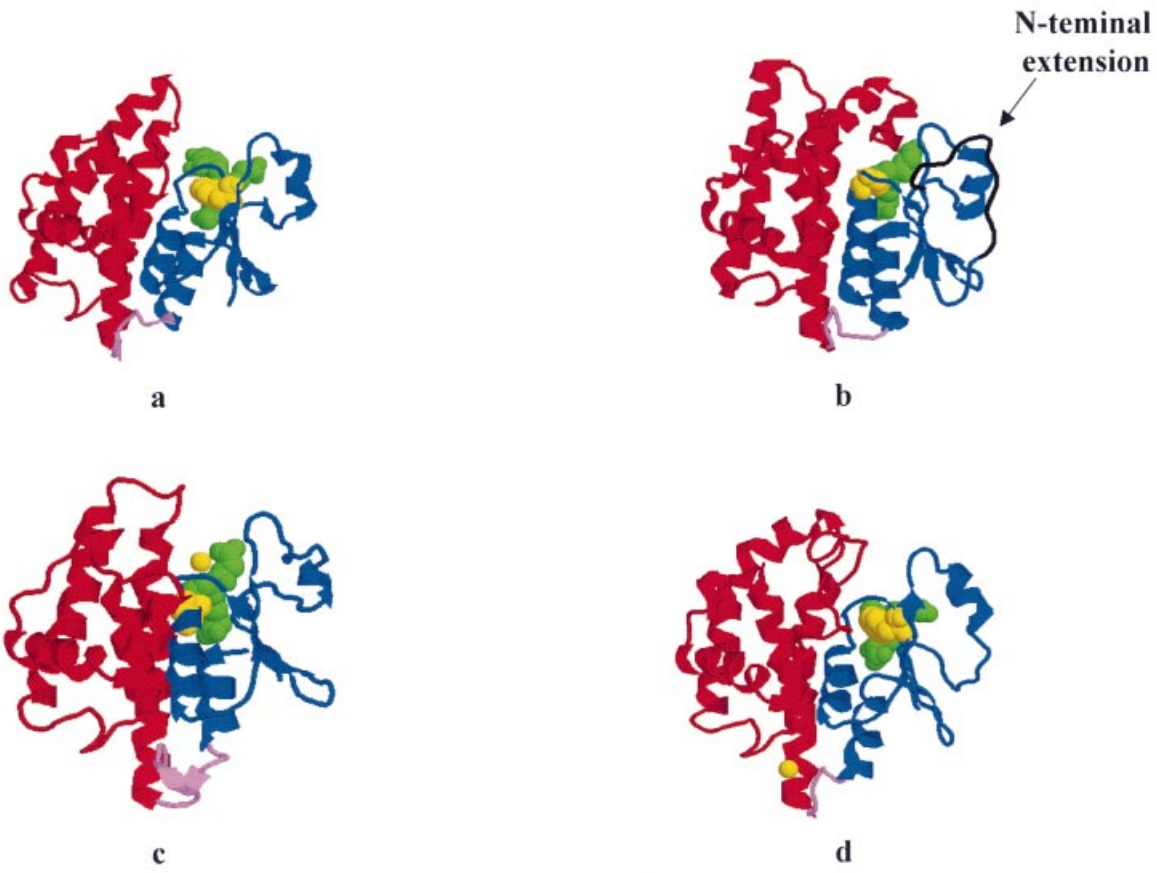

Figure 3 Domain structure of GST subunits

Three-dimensional structures of individual GST subunits are shown. The N-terminal domain 1 is coloured blue, while the C-terminal domain 2 is red. Catalytically essential residues (tyrosine in **a** and **d**; cysteine in **b** and **c**) are coloured yellow and presented in space-filling mode, while ligands with which the protein was co-crystallized are shown in green. Linker strands connecting the two domains are shown in violet. Protein database codes and references are given in parentheses: (**a**) squid Sigma class (1GSQ; [40]); (**b**) human Omega class (1EEM; [39]) [the C-terminal extension (residues 1–19) unique to this class is shown in black]; (**c**) bacterial (*Proteus mirabilis*) Beta class (1PM7; [41]); (**d**) *Fasciola hepatica* Mu class (1FHE; [84]). A multi-media adjunct to this Figure can be viewed at http://www.BiochemJ.org/bj/360/bj3600001add.htm

which includes residues contributing to the GSH binding site; for example, Thr-68 forms two hydrogen bonds with the α-carboxylate group of the glutamate residue of GSH via its –OH group and amide nitrogen [80].

Some important points of difference between the Alpha/Mu/Pi structures include the fact that, while GSH is stabilized in the GSH binding site by interactions such as hydrogen bonding and salt bridges in structures from all three classes, only the interaction with the conserved aspartic acid residue of α-4 in domain 2 is common to all structures [2,80]. Moreover, each of the Mu and Alpha classes have a unique structural feature which later structures from the same classes suggest are class-specific. The Mu-class enzymes have a characteristic 'Mu loop' which is located between β-2 and α-2 [51]. This results in a deeper active-site cleft than is found in Pi-class GSTs [3]. Alpha-class domain 2 contains an extra α-helix (α-9) which packs on to the hydrophobic binding site, thus resulting in a smaller and more strongly hydrophobic site compared with the larger, more open sites of the Mu and Pi classes [80]. This helix is thought to be important to dimer stability and the binding of non-substrate ligands to Alpha-class GSTs [93], and affects both the rate of GSH binding and the ionization state of the catalytically essential residue Tyr-9 [94].

THETA CLASS

Mammalian Theta-class GSTs were originally overlooked because they did not bind to affinity matrices such as GSH–agarose and *S*-hexyl-GSH–agarose, but were eventually discovered to be distinct from the other three classes [95]. This class shows only 7% overall sequence identity with the Alpha/Mu/Pi classes [43] and has unique substrate specificity, in that Theta-class enzymes lack activity with 1-chloro-2,4-dinitrobenzene (CDNB), the 'universal' GST substrate. Moreover, unlike enzymes in the Alpha/Mu/Pi classes, Theta-class GSTs have a catalytically essential serine rather than a tyrosine in the region of the N-terminus (Figure 5) [50,83,96,97]. It has been suggested that genes for enzymes similar to those in the Theta class are widespread in nature, being found in bacteria, yeast, plants, insects and other sources [43]. It was later found that some Theta-class GSTs are active with CDNB as substrate (e.g. that from broccoli [98]), excluding the lack of this activity as a definitive classification criterion. Two distinct homodimers (hGST T1-1 and hGST T2-2) have been identified in humans, with the T1 and T2 subunits showing only some 50% sequence similarity [99,100]. A null phenotype at the T1 locus in humans occurs in 10–38% of various ethnic groups which, as with the Mu class,

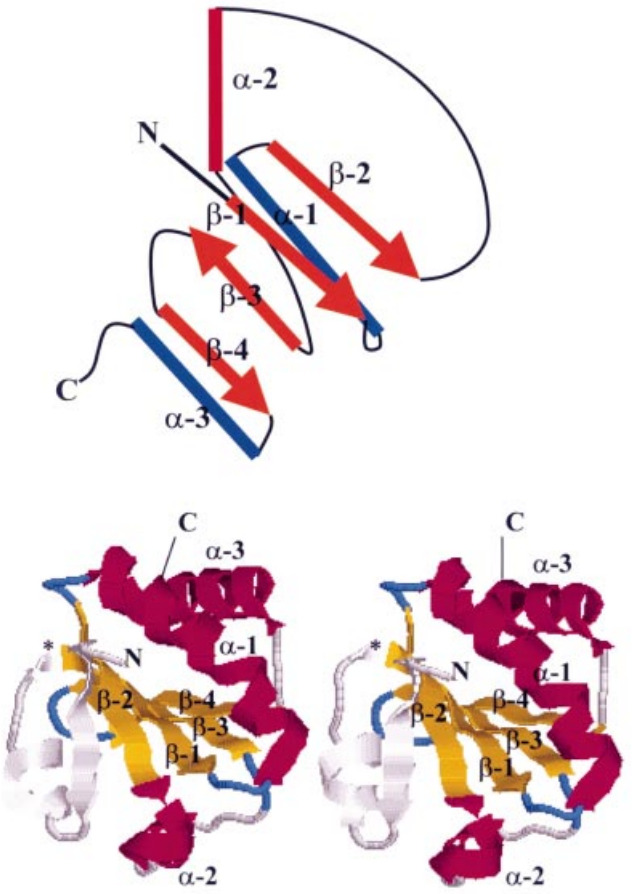

Figure 4 The thioredoxin fold

A schematic diagram representing the thioredoxin fold is shown above a RasMol depiction of the thioredoxin dimer [85]. In the diagram, α-helices are shown as cylinders, while β-sheets are shown as orange arrows. The four β-sheets are essentially co-planar, with one helix (α-2) shown in red above this plane and the other two α-helices (α-1 and α-3) shown in blue below the plane. The *cis*-Pro loop links α-2 to β-3. In GSTs, domain 2 is connected to the C-terminus by a short linker peptide. In thioredoxin itself, β-sheets are coloured yellow, while α-helices are magenta. The thioredoxin fold has an extra β-sheet and α-helix at the N-terminus (residues 1–21) ending at the point denoted by * where the fold proper begins. These additional N-terminal features are coloured grey. A multi-media adjunct to this Figure can be viewed at http://www.BiochemJ.org/bj/360/bj3600001add.htm

may possibly underlie an increased risk of toxicity in response to certain xenobiotics [57,70,71,99,101–103].

Substrate affinity has been used to distinguish Alpha/Mu/Pi GSTs from those in the Theta class. By comparing the ratio between the K_m and the ambient GSH concentration, it has been suggested that Alpha/Mu/Pi-class GSTs may have evolved towards product retention at the expense of catalytic efficiency. By contrast, Theta-class rGST 5-5 displays a K_m/ambient substrate concentration ratio similar to the range of values for glycolytic enzymes [49]. Interestingly, studies of proton release during catalysis confirmed that Alpha/Mu/Pi GSTs may activate GSH by a mechanism different from that of the Delta-class enzyme from the Australian blowfly (*Lucilia cuprina*) [104,105], which was originally assigned to the Theta class [50].

Crystal structures are available for Theta-class GSTs from human [83], insect (*L. cuprina*) [50] and a plant species (*Arabidopsis thaliana*) [96]. Unlike in the Alpha/Mu/Pi enzymes, the mainly hydrophobic inter-subunit interface lacks a 'lock and key' motif, and the cleft between the two subunits is significantly less pronounced in this class (Figure 2). With the *L. cuprina* structure as a guide, sequence comparisons revealed a consensus pattern of residues concentrated in the α-2 helix which appeared to be common to Theta-class GSTs and which was distinct in all the classes for which structures were then available [106]. Using this consensus sequence to scan sequence databases, several stress-related proteins were suggested to be related to the Theta-class GSTs. This analysis also included unexpected plant proteins in the Theta class, such as a soyabean lactoylglutathione lyase (EMBL Data Library accession no. S47177; B. Koellner, B. Finkelnberg, R. Mayerbacher, C. Paulus and R. Springer), a pathogenesis-related protein from potato [108] and a multi-stress protein from the tobacco plant [109]. Several plant GSTs originally assigned to the Theta class are now assigned to other classes [7,110]. While the Theta class active site shows replacement of the catalytically essential tyrosine found in the Alpha/Mu/Pi classes with a serine [50,97], hGST T2-2 has been demonstrated to act as a sulphatase with menaphthyl sulphate to generate menaphthyl-GSH and free sulphate. The active-site serine is not essential for this reaction, suggesting that the architecture of the active site makes a significant contribution to catalysis in this enzyme [111].

Interestingly, the analysis of Rossjohn et al. [106] did not find migration inhibitory factors (MIFs) to be included in the Theta class, despite the facts that this protein is known to bind GSH [112] and that antibodies to MIF cross-react with Theta-class GSTs [46]. Sequence alignments show that MIF lacks the proline residue responsible for the *cis*-Pro loop which is conserved in all GSTs, and shows replacement of the catalytic serine with a threonine. The most likely explanation for these findings is that MIF and Theta-class GSTs may have evolved from a common ancestral gene, but that there is no longer a close sequence or three-dimensional relationship between the two groups of proteins, although some residual topological and functional similarity remains [106]. It is striking in this example that immunological criteria include MIF in the Theta class, while structural criteria exclude it.

In constructing a phylogenetic tree for the evolution of Zeta-class GSTs (see below), the *L. cuprina* sequence was re-classified to an insect-specific Delta class [45]. It is clear from this tree that the Delta and Theta classes evolved from a common ancestor, and not one from the other. While showing overall structural similarity with Theta-class enzymes, Delta-class enzymes, in addition to primary structural differences, can bind to GSH–agarose (unlike many Theta-class GSTs) and lack a C-terminal helix which packs over the active site of Theta-class GSTs [83]. The significance of the Delta class is discussed further below, in the context of the classification of insect GSTs.

KAPPA CLASS

As stated above for plant GSTs, a number of enzymes originally allocated to the Theta class were subsequently re-assigned to separate classes. For example, a GST isolated from the rat mitochondrial matrix and consisting of a homodimer of subunit-13 was originally assigned to the Theta class [113]. However, full-length sequence data of a cDNA clone coding for this enzyme revealed that it has a number of unique features which merited placing it in a separate class, called the Kappa class [44]. The most important of these is that this sequence lacks the SNAIL/TRAIL motif found in all other GST classes. Northern blots suggest that there is a single gene copy of this enzyme, and orthologues have also been found in human and pig [44]. A three-dimensional structure is not yet available for this class.

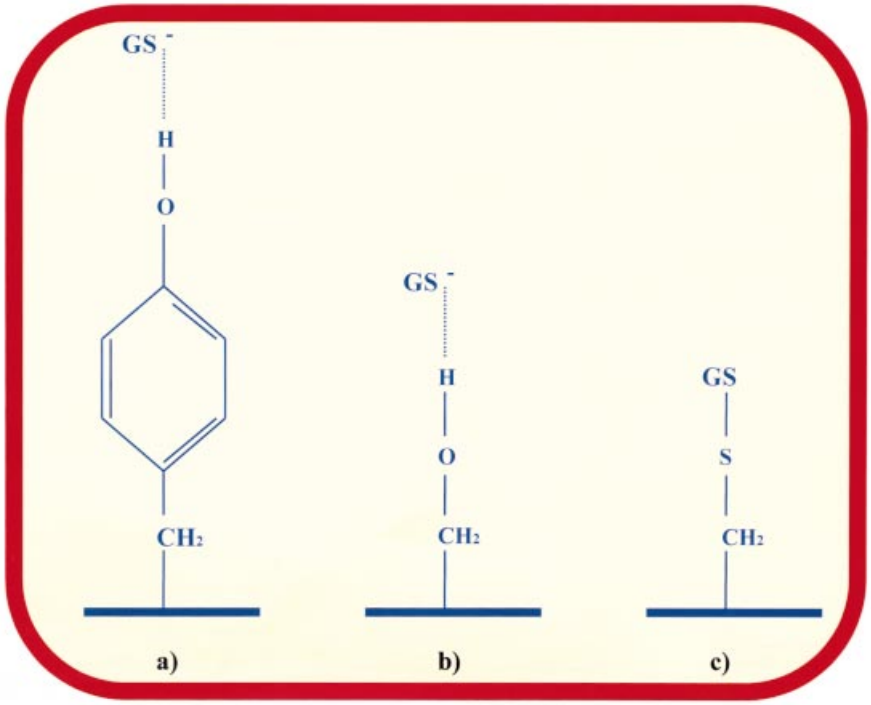

Figure 5 Active-site residues in GSTs

In most GST classes, an N-terminal tyrosine residue (**a**) interacts with GSH to stabilize the thiolate anion, with a consequent decrease in pK_a. In the Theta and possibly the Zeta classes, this role is carried out by a serine residue (**b**), while in the Omega and Beta classes a mixed disulphide is formed with a cysteine residue (**c**).

OMEGA CLASS

Screening of a human EST sequence database identified a novel GST the crystal structure of which shows several marked differences compared with the previously described mammalian GST classes [39]. In particular, the enzyme has an unusual 19–20-residue proline-rich N-terminal extension not present in other classes which, together with the C-terminal domain, forms a unique structural unit, while domain 2 contains seven α-helices (Figure 3). The inter-subunit interface is dominated by non-polar interactions and was found to be considerably more open than in other GSTs; the buried area is only 1960 Å^2, compared with the more usual values of 2700–3400 Å^2 found in most other GST dimers. Interestingly, the buried area observed for plant Theta-class GSTs (which also have a particularly open dimer interface) was 2370 Å^2 [96]. In the active site there was found to be interaction between cysteine-32 and the −SH group of GSH, which contrasts with the situation in other mammalian GSTs, where either a tyrosine (Alpha/Mu/Pi) or a serine (Theta) performs this function (Figure 5). The bacterial Beta class (see below) also features an active-site cysteine which forms a mixed disulphide with GSH [41].

This enzyme shows particularly high activity with CDNB, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, *p*-nitrophenyl acetate and thiol transferase, but relatively modest dehydroascorbate reductase activity. The enzyme appears to be expressed in most human tissues, and orthologues exist in mouse [114], rat [115] and the nematode *Caenorhabditis elegans* [39]. Like the examples mentioned above, the mouse orthologue was originally assigned to the Theta class [114].

A possible 'housekeeping' function of Omega-class GSTs may be to act as a GSH-dependent thiol transferase, removing S-thiol adducts which some proteins form with GSH and cysteine in response to oxidative stress. This may be facilitated by the particularly open hydrophobic substrate binding site, which is large enough to accommodate a polypeptide chain [39]. Sequence similarity between Omega-class GSTs and a nuclear chloride channel, CLIC1, prompted investigation of whether this enzyme can form or modulate ion channels [116]. This revealed that, while hGST O1-1 does not itself form ion channels, it inhibits cardiac muscle ryanodine receptor (RyR2) activity, while skeletal muscle ryanodine receptor (RyR1) activity is potentiated. Ryanodine receptors are calcium channels which occur in the endoplasmic reticulum of various cells. The inhibition effect depends on the GST being catalytically active, and mutation of cysteine-32 to alanine abolished it. This study [116] suggested a possible novel role for Omega-class GSTs in protecting cells from apoptosis induced by Ca^{2+} mobilization from intracellular stores.

NON-MAMMALIAN GSTs

A phylogenetic analysis of 63 available GST-like sequences (several of which were also from stress-related, non-GST proteins) revealed that, if class-assignment criteria were chosen to maintain the traditional mammalian classes (at that time: Alpha, Mu, Pi, Theta, Sigma and MAPEG), then these sequences could be distributed among 24 and 44 individual classes [117]. Despite limitations of data available for a more comprehensive comparison, this study suggested that there may be many more GST classes yet to be discovered, and this indeed proved to be the case with the Omega and Kappa classes, as has just been described. In addition, several new classes have been discovered in non-mammalian species. Orthologues of these new classes of

genes and proteins were later often found to be present also in mammals, in some cases carrying out functions not ascribed previously to GSTs.

SIGMA CLASS

Based on alignments of 71 sequences, a new class of GSTs was designated as Sigma [118]. This class includes GSTs purified from squid [119,120] which also show 42–44% similarity with cephalopod S-crystallin, the major protein of eye lens [121]. Determination of the three-dimensional structure revealed unique features, especially at the dimer interface and in the active site [40]. The distinctive hydrophilic dimer interface – there is no 'lock and key' motif as in the Alpha/Mu/Pi classes – plays a key role in protein stability in this group of enzymes [122], and electrostatic interactions near the active site appear to be especially important in this regard [123]. This is not a defining feature of this class, however, as the haematopoietic prostaglandin D synthase, which is also a Sigma-class GST (see below), has a hydrophilic interface with a lock and key motif which is similar to that found in the Alpha/Mu/Pi classes [52].

The active site of Sigma-class GSTs binds GSH in a similar manner as in the Alpha/Pi-class GSTs, while the mammalian Mu-class enzyme and that of *Schistosoma japonicum* (see below) provide a different set of interactions with the tripeptide in the active site [40]. A third binding site for GSH conjugates, which may be important in conjugate transport, has also been identified [124]. A structure for S-crystallin determined by homology modelling suggests that lack of GST activity in this protein may be due to insertion of 11 residues between the conserved α-4 and α-5 helices. This results in a very closed conformation around the putative active site, despite overall topological similarities with the Sigma-class structure [125].

Sigma-class enzymes have been strongly implicated in prostaglandin synthesis. A relationship between Sigma-class GSTs of helminths and a GSH-dependent prostaglandin-H E-isomerase was revealed by comparison of partial sequence data [126]. This enzyme was later purified from the parasitic nematode *Ascaridia galli* and confirmed to be a Sigma-class GST [127]. It is thought that this enzyme promotes the endogenous synthesis of prostaglandin E in multicellular parasites. In vertebrates, homologues which are also Sigma-class GSTs and which display high prostaglandin D synthase activity have been identified in rat, mouse, chicken and human [52,127,128]. Two types of prostaglandin synthase are known in mammals, and are designated L-PGDS and H-PGDS [129]. The former enzyme is dependent on lipocalin and is expressed mainly in brain, while the latter is dependent on GSH and is expressed mainly in peripheral tissues (Figure 1). Remarkably, sequence alignments show no similarity between L-PGDS and H-PGDS, while the latter is highly similar to Sigma-class GSTs [52]. Moreover, the crystal structure of the rat enzyme shows a prominent active-site cleft which accommodates bifunctional activity (i.e. GST and prostaglandin synthase [52]). This prostaglandin synthase is thought to have originated from the same ancestral gene as Sigma-class GSTs, as suggested by common intron/exon boundaries [130].

ZETA CLASS

A phylogenetically highly conserved class of GSTs, designated the Zeta class, was also initially identified by sequence alignment [45]. This class is present in a spectrum of species ranging from plants [131] to humans [45,67]. The extent of sequence conservation [e.g. 38% between carnation (*Dianthus carophyllus*) and human, and 49% between *Caenorhabditis elegans* and human] is a particularly striking feature of this GST class [45]. The human enzyme has low GSH-conjugating activity with CDNB, ethacrynic acid and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, modest GSH peroxidase activity with both cumene and t-butyl hydroperoxides, and is implicated in detoxification of the carcinogen dichloroacetic acid to glyoxylic acid [132].

Interestingly, this enzyme is also irreversibly inactivated by dichloroacetic acid in rats, mice and humans by a mechanism in which the xenobiotic is biotransformed into a reactive metabolite [133]. Zeta-class GST is identical with maleylacetate isomerase, an enzyme of the catabolic pathway for tyrosine [67,134]. This enzyme converts maleylacetoacetate into fumarylacetacetate, and maleylacetone into fumarylacetone (Figure 1). Both of these substrates are alkylating agents, and it has been shown that inactivation of Zeta-class GST by dichloroacetic acid results in the accumulation of maleylacetone, while treatment of dialysates with this compound gives a dose-dependent inactivation of Zeta-class GST [135]. Thus the dichloroacetic acid-induced inhibition of tyrosine metabolism may explain the toxicity of this enzyme to rodents and humans [133]. As with the Alpha, Mu, Pi and Theta classes, polymorphisms in the human population have been identified in this class, which may underlie inter-individual differences in the ability to metabolize dichloroacetate and fluoroacetate [133,136].

A crystal structure for human Zeta-class GST (maleylacetoacetate isomerase) is now available [137]. While the overall fold of this protein is generally similar to the other GST classes, major differences are found in and around the α-2 helix which result in particular in a very small, almost buried, polar active site. This explains the poor catalytic activity of this enzyme with most GST substrates. The V-shaped dimer interface is lacking; in this regard, Zeta resembles the Theta and Beta (see next section) classes. This interface is dominated by hydrophobic interactions between small aliphatic residues, and a methionine residue acts as a key in a 'lock and key' motif linking the dimer together. This contrasts with other classes, which either lack this motif altogether or else feature an aromatic residue as a key. It is unclear from this structure which N-terminal residue is catalytically essential. However, serine-14 is close enough to the GSH thiol to interact with it, although its hydroxy group is pointing away from this group in the structure. It is possible that the protein undergoes a conformational change to allow this residue to interact with GSH, as has been found previously with Pi-class GST [138].

BETA CLASS

GSTs have been described in a wide variety of bacteria, including *Escherichia*, *Proteus*, *Pseudomonas*, *Klebsiella*, *Enterobacter Serratia*, *Burkholderia* and *Rhodococcus* species (reviewed in [139]). In addition to the role of GSH conjugation in antibiotic resistance [29], these enzymes are also able to bind antibiotics [140]. Determination of the three-dimensional structure of the well-characterized enzyme from *Proteus mirabilis* revealed a mixed disulphide between a conserved cysteine residue and the thiol group of GSH, which is similar to that observed in the mammalian Omega class (Figure 5) [39,41]. Sequence analysis suggested that a number of other bacterial GSTs share this cysteine, which led to designation of a new GST class, Beta (from bacteria), most members of which would originally have been allocated to the Theta class. Comparison of the *P. mirabilis* structure with those from Theta-class plant (*A. thaliana*) and Delta-class insect (*L. cuprina*) sources revealed important differences at the dimer interface: this is close-packed and dominated by polar interactions in the bacterial structure,

whereas the Theta and Delta classes have a more open, V-shaped interface dominated by hydrophobic interactions. Site-directed mutagenesis has demonstrated that none of the cysteine, tyrosine or serine residues at the N-terminus are catalytically essential, but that C-terminal histidine-106 and lysine-107 may contribute to the interaction with GSH [141]. There may be more than one GST class in bacteria, since site-directed mutagenesis and sequence alignments suggest that the GST from *Ochrobacterium anthropi* has a conserved active-site serine, as in the Theta and Zeta classes [140].

FUNGAL GSTs

By comparison with other major groups, such as mammals, plants and insects, relatively little is known about GSTs from fungi. It is clear that these enzymes are expressed widely in a large number of fungal species, although not in *Saccharomyces cerevisiae* [142]. As with mammals, plants and insects, the enzymes are expressed in multiple forms which appear to be inducible by xenobiotics [143,144]. Full-length sequences have only been published for the two GSTs of *Issatchenkia orientalis* [143] and for the recombinant enzymes of *S. cerevisiae* [145]. The sequences of *I. orientalis* GSTs Y-1 and Y-2 are quite distinct from those of the Alpha/Mu/Pi GST classes, but show limited similarity with the N-terminal region of several Theta-class enzymes [143]. GST Y-1 shows conservation of the N-terminal catalytically essential serine of the Theta class, but in GST Y-2 this is replaced with a threonine, which may explain this enzyme's significantly lower catalytic activity compared with GST Y-1 [143]. Comparison of partial N-terminal sequences and immunoblotting suggest that GSTs from *Phanerochaete chrysosporium*, *Mucor circinelloides* and *Yarrowia lipolytica* are also similar to those in the Theta class; however, in the absence of full-length sequence data, such allocations cannot be definitive [144,146,147].

Two GST genes have been cloned and sequenced from *S. cerevisiae* and their recombinant proteins studied [145]. These enzymes show little similarity to mainstream GSTs, although their N-terminal sequence shows moderate similarity to GSTs Y-1 and Y-2 of *I. orientalis*, to GST-III and GST-IV of maize (see next section) and to other *S. cerevisiae* proteins, such as the product of *URE2* [148], elongation factor 1γ [92] and the a-agglutinin protein [149]. It is noteworthy that a GST purified from *Mucor circinelloides* also showed some N-terminal sequence similarity to the a-agglutinin protein [144]. While the recombinant *S. cerevisiae* enzymes are membrane-bound in the endoplasmic reticulum, they function as dimers, suggesting little similarity to microsomal GSTs (which are trimeric).

PLANT GSTs – PHI AND TAU CLASSES

Since Theta-class GSTs show generally lower amino acid sequence identities than in other classes, many plant GSTs were at first allocated to this class. Based on sequence alignments of full-length sequences, plant enzymes were originally divided into three distinct groups, designated I, II and III [68,150,151]. This was intended to be an analagous classification system to the Alpha/Mu/Pi/Theta/Sigma system used for mammalian GSTs up until then. Type I included GSTs with herbicide-detoxifying activities, for which the genes contain three exons [152]. Type II GSTs are now thought to be related to the Zeta class, and their genes contain 10 exons. Type III consists of GSTs which are auxin-inducible; their genes contain two exons [110]. An extension of this classification into Type IV was then proposed for a group of *A. thaliana* GSTs which are closely related to the mammalian Theta class [28]. Based on sequence comparisons, it was suggested that plant GSTs could be classified into two classes: the previously described Theta class and a novel plant-specific class designated Tau [110]. However, some plant GSTs were found not to fit readily into either the Theta or Tau classes (e.g. carnation gst 1 [153,154]). The current classification system recognizes four main classes, two of which are plant-specific and two of which are more phylogenetically widespread. The plant-specific classes are Phi (previously Type I) and Tau (previously Type III). The Theta class includes enzymes previously designated as Type IV, while the Zeta class includes those previously classified as Type II [7].

GSTs play many roles in plants [7,68], having been implicated in herbicide resistance [155–158], being inducible by pathogens and/or dehydration [108], showing direct binding of auxins [159] and catalysing the formation of anthocyanins [68,69,160]. Moreover, they appear to be important in both higher and lower plant species [161]. In the case of wheat, it has been shown that the existence of high levels of GST is not a result of domestication, but is characteristic of progenitor species from which domestic strains were bred [162]. Unlike animals, plants appear to have made the transition from water to land only once and to have evolved the entire multicellular diploid phase of their life cycle on land [163].

As in mammals, it is known that GSTs from several classes are expressed in most plant species, there are multiple genes for GSTs in most species and GSTs are abundantly expressed [110,160]. Many plant GSTs are naturally expressed as heterodimers, but when their genes are cloned into bacteria they form homodimers more readily. A method for expressing such heterodimers in *E. coli* has been developed [164]. Largely for economic reasons, the best-studied GSTs are those from maize (*Zea mays*) and the weed *A. thaliana*.

Three-dimensional structures are available for *A. thaliana* GST [96], for maize GST-I in complex with lactoylglutathione [165] and atrazine–GSH [166], and for maize GST-III in its apoenzyme form [167]. On cloning and sequencing maize GST-III for crystallization, a number of errors in previously reported primary structures were discovered, and these have now been corrected [167]. In all three plant GSTs the N-terminal domain and mainly hydrophobic inter-subunit interface are quite similar, and differences are concentrated in the C-terminal domain where a hydrophobic substrate is likely to bind, which is considerably broader in plant than in mammalian enzymes [96,165]. Comparison of the structure of GST-I complexed with herbicide with that of GST-III provided the first evidence for 'induced fit' in GSTs, since a loop consisting of residues 36–45 of GST-I moves to make contact with the ligand [165]. The three plant GST structures have provided plausible explanations for the selectivity of maize GSTs for herbicides [158,166].

In maize, a GST encoded by the *Bz2* gene was found to conjugate GSH to cyanidin 3-glucoside, the final step in the biosynthesis of anthocyanins [68]. These compounds share a common core structure, which may be modified by hydroxylation, methylation, glycosylation or acylation in a species-specific manner to give a range of red, blue and purple flower colours. This conjugate is transported into intracellular vacuoles by a GS-X pump which is similar to those of mammalian cells (Scheme 1) [8]. This pump is located in the vacuolar membrane of both plant and yeast cells [168,169]. Studies with other species suggest that this process is widespread and may provide a biotechnological means of introducing novel colours into flowers [69,170,171]. Anthocyanin synthesis may be regarded as yet another endogenous function which has evolved due to the multiplication and diversification of GST genes (Figure 1) [160].

INSECT GSTs – CLASSES I AND II

GSTs have been purified from more than 24 individual insect species and, as with plants and mammals, the enzymes are expressed at high levels, in multiple isoenzyme forms and in different patterns at various developmental stages [172,173]. GSTs have been implicated in resistance to insecticides and are often induced in resistant strains [26,27,47,174–176]. For example, in many insects dehydrochlorination catalysed by GST is the major mechanism responsible for resistance to 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT) [177], and GSH conjugation is thought to be a secondary mechanism for resistance to some organophosphorous insecticides [178].

The most generally accepted classification system for insect GSTs recognizes two immunologically distinct classes designated I and II [47,179]. Class I GSTs have been identified in *Musca domestica*, *Drosophila melanogaster*, *Anopheles gambiae* [27], *Anopheles Dirus* ([180] and references therein) and *L. cuprina* [50] and, based on sequence alignments, are most closely related to enzymes in the mammalian Theta class [27,45,117], although they have now been re-classified to an insect-specific Delta class [45]. Insect class I GST genes are generally intronless in the coding region, but may be interrupted by introns in a 5′ untranslated part of the gene [173,181]. Insect GST genes exist in complex orientations on chromosomal clusters, and multiple enzymes may be produced as a result of alternative mRNA splicing [182].

Class II GSTs have been identified in *Manduca sexta* and *D. melanogaster* (see literature cited in [180]) and, based on published phylogenetic trees, are thought to be similar to enzymes of the Sigma class [52,117,183]. A more recent BLAST search of the newly available *D. melanogaster* genome found no candidate genes for the Alpha/Mu/Pi classes, but identified several members of the Omega, Zeta, Sigma and Theta classes which had been included previously in insect class II [184]. It is likely, therefore, that the existing classification system can be extended to insects by designating class I GSTs as Delta class and allocating class II GSTs to the Omega/Zeta/Sigma/Theta classes.

HELMINTH GSTs

Helminth organisms such as *Fasciola hepatica*, *Schistosoma mansoni* and *Ascaris lumbricoides* are prominent parasites affecting human and animal health. These organisms are unusual in that they have generally low levels of phase I and other detoxification enzyme activities, but express GSTs, especially in response to drug treatment [185]. GSTs are therefore tempting targets both for chemotherapy and for vaccine development. It has already been pointed out that a helminth GST has been allocated to the Sigma class [126]. However, other GSTs have been identified in organisms of the genuses Ascaris, Schistosoma and Fasciola. Two of these GSTs – those from *Schistosoma japonica* [186,187] and the liver fluke *F. hepatica* [84] – have been crystallized.

A. lumbricoides is a nematode worm that is thought to infect more than one billion people worldwide. While not in itself immediately fatal, it can have debilitating effects, especially in children, which can lead to serious clinical conditions. A GST has been cloned and sequenced from *Ascaris suum* [188]. While showing little direct sequence similarity to the mammalian Alpha/Mu/Pi-class GSTs (28–37 %), a three-dimensional structural model suggests that this enzyme most closely resembles Pi-class enzymes, as it lacks both the Mu loop and the extra α-helix of the Alpha class. The low level of sequence identity has facilitated the production of polyclonal antibodies specific for this enzyme [188].

Schistosoma are helminths that cause a parasitic infection called schistosomiasis that affects more than 275 million people worldwide. Schistosomal GSTs have been studied intensively because they are believed to be responsible for resistance to the major drug used in treatment of this disease, praziquantel. A three-dimensional structure for the *S. japonicum* GST fused with a short epitope from human HIV1 was determined by molecular replacement using the co-ordinates for the rat Mu-class structure [51]. This structure closely resembled the Mu-class enzyme, especially in domain 1 [186].

The structure of a non-fusion schistosomal GST, both complexed and non-complexed with praziquantel, was then determined [187]. This structure demonstrated that a single praziquantel molecule binds to a non-substrate ligand binding site. This is located in the inter-subunit cleft, and the manner of binding is such that the ligand can occupy only one of two possible overlapping and symmetry-related binding sites in the cleft. This is reminiscent of the binding of a steroid affinity label to mammalian Alpha-class rGST 1-1 [23]. The binding site is approx. 9 Å from the active-site tyrosine, suggesting that drug binding is a more likely mechanism of praziquantel resistance in this organism than conjugation with GSH. The overall fold of the protein resembles that of mammalian Mu-class GSTs, with the exception of an eight-residue insertion compared with the rat sequence which creates a more open hydrophobic substrate binding site. Points of structural difference such as these are key targets in vaccine development in helminths.

Four distinct GSTs (Fh-1, -7, -47 and -51) have been cloned from the liver fluke *F. hepatica* which may be distinguished from each other on the basis of substrate specificity, inhibitor sensitivity [189] and immunoblotting [48]. A crystal structure of GST Fh-47 was determined which confirmed overall resemblance to the Mu class in general and to the GST of *S. japonicum* in particular (Figure 3) [84]. Molecular modelling of the other three enzymes based on this structure showed critical differences in the xenobiotic substrate binding site, which may explain substrate/inhibitor differences between these isoenzymes as well as differences in the non-substrate binding site. Surprisingly, the most immunogenic epitopes in Fh-47 [48] were found not to be in the most solvent-exposed region of the protein.

From the perspective of classification, it is interesting that, whereas helminth organisms express GSTs that are clearly related to the Mu, Pi and Sigma classes of other organisms, these enzymes contain sufficient regions of structural difference compared with host enzymes to hold out the prospect of the development of parasite-specific vaccines.

POSSIBLE EVOLUTIONARY SIGNIFICANCE OF GST CLASSIFICATION

Drug detoxification enzymes have existed in both prokaryotes and eukaryotes for more than 2.5 billion years [190,191]. GSTs constitute a very ancient protein superfamily that is thought to have evolved from a thioredoxin-like ancestor in response to the development of oxidative stress [86,92]. Other GSH- and cysteine-binding proteins share the thioredoxin-like fold [86], and it is increasingly becoming clear that GSTs share sequence and structural similiarities with several stress-related proteins in a wide range of organisms [106]. It is thought that the multiple GST classes arose by a process of gene amplification followed by divergence, perhaps involving a mechanism similar to DNA shuffling, resulting in novel catalytic activities [192,193]. Many workers have used sequence comparisons to generate phylogenetic trees to identify likely patterns of divergence. Ideally, it ought to be possible to compare all full-length sequences known

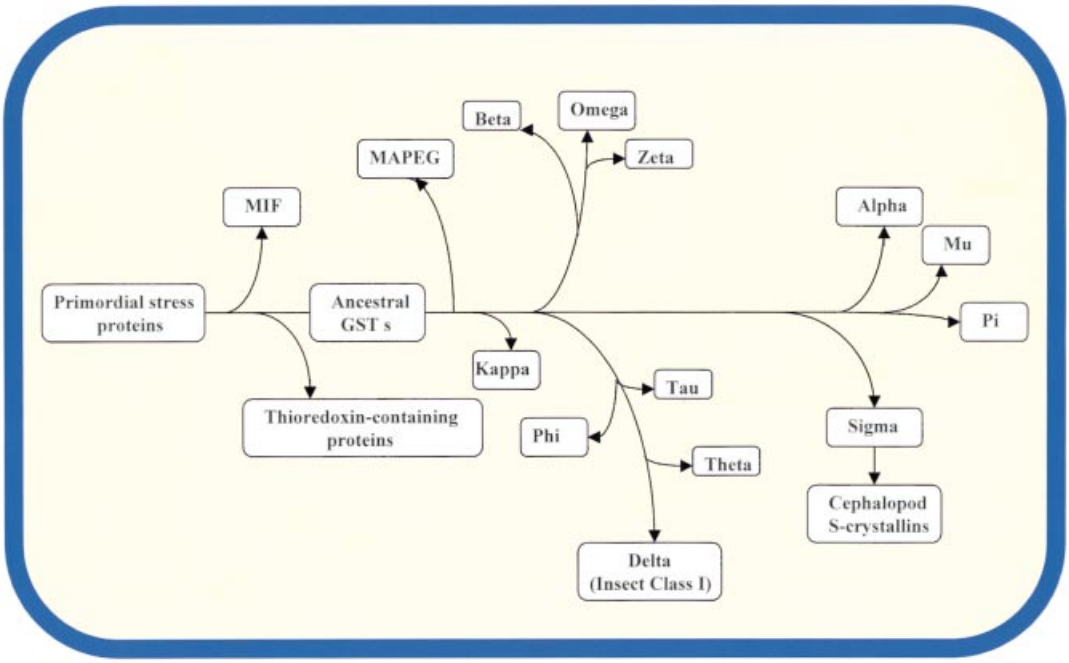

Figure 6 Possible pattern of divergence in the GST superfamily

Based on sequence alignments described in [39,52,117], a plausible pattern of divergence resulting in multiple GST classes is shown.

to code for GSTs, but in practice a subset of sequences is usually used to avoid misleading results [117]. In making alignments, therefore, it is necessary to select a subgroup of GSTs, and this accounts for the slight differences, for example, between trees published in [39,52,117].

Protein families arise as a result of duplication and divergence of entire coding regions, independently folding domains or short sequence motifs that can be inherited in proteins with sometimes quite different functions to the primordial ancestor [194]. As has been pointed out in the discussion of individual GST classes, several non-GST proteins have been implicated as being related to GSTs by sequence alignment [106,117] or based on immunoblotting [46]. Examples include bacterial stringent starvation proteins, plant pathogen/stress resistance proteins and the URE2 protein from *S. cerevisiae* ([106] and references therein), eukaryotic translation elongation factor 1γ [92] and MIF [46,112]. These relationships suggest that a common stress-related ancestor may have pre-dated the evolution of the thioredoxin fold. The classes described in this review reflect groups of GSTs that diverged at various points during evolution, and it is clear that these are now widespread in both mammalian and non-mammalian species. In cases where only a small number of GST classes have yet been described, it is possible that other classes exist which have not yet been discovered and which may carry out novel functions not necessarily associated with detoxification (Figure 1). A schematic diagram summarizing a plausible pattern of divergence is shown in Figure 6.

CONCLUSIONS

Sequence comparisons confirm that GST classes exist in a surprisingly widespread range of organisms. Some of these classes were originally discovered in non-mammalian species and were only later found in mammals. This wide taxonomic distribution tends to confirm that GSTs play a fundamental role in protection against endogenous or exogenous toxic chemicals. Moreover, a growing number of non-detoxification functions have now been attributed to GSTs, as exemplified in Figure 1. On the other hand, a number of groups have identified sequence, tertiary structure or immunological relationships between GSTs and a surprisingly wide range of stress-related and other proteins in bacteria, yeasts, plants, etc. Despite low levels of overall sequence identity, three-dimensional structures confirm a very similar overall fold in GST subunits, with class-specific features found especially in the active site, inter-subunit interface and domain 2. Some of the most informative three-dimensional structures have been obtained for GSTs from non-mammalian species, and these have helped to define the range of structures possible for the superfamily as well as underlining invariant features.

It has been suggested that more GST classes exist than have yet been described [117]. In the case of plant GSTs it has been proposed that the enzymes discovered so far may be a reflection of particular research interests in aspects of plant metabolism, such as auxin binding and herbicide resistance, but that other GSTs may exist that have simply not yet been discovered [110]. Undoubtedly much GST research has focused on resistance phenomena, as outlined in this article. However, as exemplified by the discovery of the first mammalian Sigma-class GST as a haematopoietic prostaglandin D synthase [52], it is possible that far more GSTs or GST-related genes exist than was previously suspected. Application of MS methods have already revealed several new isoenzymes in previously well-researched mammalian sources [61,195,196]. The availability of large-scale genomic and EST databases holds out the prospect of the discovery of novel GST classes with an even wider range of structures and functions than is now known. Such studies will undoubtedly also help to clarify further the complex relationships between stress-related proteins and this complex and ancient enzyme superfamily.

Note added in proof (received 15 October 2001)

A recent publication by Ranson et al. [197] describes a third insect-specific class of GSTs that appears to be widespread in insects and which plays a major role in DDT resistance in *A. gambiae*. Their phylogenetic analysis also suggests that class II GSTs include Theta, Sigma, Omega and Zeta class enzymes as suggested previously [184].

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