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## Glutathione transferases: a structural perspective

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The glutathione transferases (GSTs) are one of the most important families of detoxifying enzymes in nature. The classic activity of the GSTs is conjugation of compounds with electrophilic centers to the tripeptide glutathione (GSH), but many other activities are now associated with GSTs, including steroid and leukotriene biosynthesis, peroxide degradation, double-bond cis-trans isomerization, dehydroascorbate reduction, Michael addition, and noncatalytic "ligandin" activity (ligand binding and transport). Since the first GST structure was determined in 1991, there has been an explosion in structural data across GSTs of all three families: the cytosolic GSTs, the mitochondrial GSTs, and the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG family). In this review, the major insights into GST structure and function will be discussed.

### Keywords

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# Glutathione Transferases: A Structural Perspective

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## Abstract

The glutathione transferases (GSTs) are one of the most important families of detoxifying enzymes in nature. The classic activity of the GSTs is conjugation of compounds with electrophilic centers to the tripeptide glutathione (GSH), but many other activities are now associated with GSTs, including steroid and leukotriene biosynthesis, peroxide degradation, double-bond *cis-trans* isomerization, dehydroascorbate reduction, Michael addition, and non-catalytic “ligandin” activity (ligand binding and transport). Since the first GST structure was determined in 1991, there has been an explosion in structural data across GSTs of all three families: the cytosolic GSTs, the mitochondrial GSTs and the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG family). In this review, the major insights into GST structure and function will be discussed.

**Keywords:** *Glutathione transferase; GST; structure; function; catalytic mechanism*

**Abbreviations:** GST: glutathione transferase; GSH: glutathione; MAPEG: membrane-associated proteins in eicosanoid and glutathione metabolism; JNK: c-Jun

N-terminal kinase; CLIC: intracellular chloride ion channels; mPGES: microsomal prostaglandin E synthase; LTC<sub>4</sub>-S: LTC<sub>4</sub> synthase; FLAP: 5-lipoxygenase-activating protein; PAH: polyaromatic hydrocarbon; BCNU: N,N'-bis(2-chloroethyl)-N-nitrosourea; EA: ethacrynic acid; PE: Phenanthrene epoxide; BPDE: 7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene; HNE: 4-hydroxynon-2-enal; MES: morpholinoethanesulfonic acid; HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPDS: hematopoietic prostaglandin D synthase; IMN: indomethacin; HHT: 12(*S*)-hydroxy-5(*Z*),8(*E*),10(*E*)-heptadecatrienoic acid.

## Introduction

Detoxification is a vital metabolic and defense capability of living organisms. The glutathione transferases (formerly glutathione S-transferases, GSTs) play a significant role in detoxification across multiple kingdoms and phyla. The key reaction catalyzed by this superfamily is conjugation of the tri-peptide glutathione (GSH,  $\gamma$ -glutamyl-cysteinyl-glycine) with a hydrophobic co-substrate possessing an electrophilic center (E.C. 2.5.1.18), although many related activities are known. Conjugation with GSH renders those compounds more soluble and substrates for cellular export proteins such as the multidrug resistance related protein (MRP1) which are ATP binding cassette (ABC) transporters (Suzuki et al. 2001). Three distinct superfamilies of GST are recognized: the cytosolic GSTs (which form the largest superfamily), the mitochondrial and the microsomal GSTs. The microsomal GSTs are integral membrane proteins, now designated as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEGs). Several GSTs utilize GSH not as substrates but as cofactors (in e.g. leukotriene biosynthesis). The roles of GSTs are many. Apart from GSH-conjugation, GSTs function in hormone biosynthesis, tyrosine degradation, peroxide breakdown, dehydroascorbate reduction and other functions (Hayes et al. 2005). Perhaps most surprising is the action of GSTP1 in the inhibition of c-Jun N-terminal kinase (JNK) signaling activity (Adler et al. 1999).

Protein sequence and structure form a basis for classification of GSTs. Among cytosolic GSTs, members of the same class possess greater than 40% amino acid sequence identity. Between classes, proteins have less than 25% sequence identity. The currently recognized classes of cytosolic GSTs (with references to representative structures) are alpha (Sinning et al. 1993), beta (Rossjohn et al. 1998b), delta (Wilce

et al. 1995; Oakley et al. 2001), epsilon (Sawicki et al. 2003), zeta (Polekhina et al. 2001), theta (Rossjohn et al. 1998a), mu (Ji et al. 1993), nu (Schuller et al. 2005), pi (Reinemer et al. 1992), sigma (Ji et al. 1995; Kanaoka et al. 1997), tau (Thom et al. 2002), phi (Reinemer et al. 1996) and omega (Board et al. 2000). The mitochondrial GSTs share a deep evolutionary relationship with the cytosolic GSTs (discussed below) and are designated class kappa. In humans, cytosolic GST isozymes in classes alpha, zeta, theta mu, pi, sigma and omega are found. Both cytosolic GSTs and mitochondrial GST form dimers; heterodimers of cytosolic GSTs have been identified containing chains belonging to the same class. The MAPEG family consists of four subgroups (I–IV). Between subgroups, protein sequences share less than 20% sequence identity. In humans, six MAPEG isozymes have been identified that belong to subgroups I, II, and IV (Jakobsson et al. 1999a). Like the cytosolic- and mitochondrial GSTs, several MAPEGs such as MGST1 catalyse conjugation of GSH to a number of electrophilic compounds. Other members additionally catalyze reactions in leukotriene and prostaglandin biosynthesis (Hayes et al. 2005).

Several GSTs are of pharmacological interest. In humans, most classes of cytosolic GST catalyze conjugation reactions with drugs and other xenobiotics (Hayes et al. 2005). Isozymes of class alpha and zeta are known to possess GSH-dependant isomerization activities and will be discussed below. The sigma-class GST known as hematopoietic prostaglandin D synthase catalyses PGD<sub>2</sub> formation from PGH<sub>2</sub> in a GSH-dependant manner. Certain of the theta-class GST isozymes possess sulfatase activity (Rossjohn et al. 1998a). The omega-class GSTs share common features with the glutaredoxins: they form mixed-disulfides with GSH and catalyse reductase reactions (Board et al. 2000). In bacteria, the beta-class GSTs are implicated in a

variety of distinct processes such as protection against chemical and oxidative stresses, antimicrobial drug resistance and in the catabolism of xenobiotics (Allocati et al. 2009).

Several non-GST proteins have been shown known to contain the topological and ligand-binding features of cytosolic GSTs. These include the intracellular chloride ion channels (CLICs) (Harrop et al. 2001; Cromer et al. 2007; Littler et al. 2010), microsomal prostaglandin E synthase type-2 (mPGES-2) (Yamada et al. 2005), yeast elongation factor 1B $\gamma$  (Jeppesen et al. 2003), and the yeast prion protein Ure2p (a nitrogen catabolite repression regulator) (Bousset et al. 2001). It is noteworthy that Ure2p shows glutathione-dependent peroxidase (GPx) activity, (a frequent adjunct activity of GSTs), but no GST activity toward the classic GST substrate 1-chloro-2,4-dinitrobenzene (CDNB). The GSH conjugating activity of Ure2p can be restored by site-directed mutagenesis (Zhang et al. 2008). The structure of Stringent starvation protein A (sspA), an RNA-polymerase-associated protein, represents a distinct class of bacterial GST-like proteins (Hansen et al. 2005). SspA lacks GST activity and does not bind glutathione.

## **General structural features of GSTs**

### *cytosolic GSTs and mitochondrial GSTs*

Because of their relevance to toxicology, cancer and drug metabolism (see reviews in this issue), the mammalian cytosolic GSTs (in particular, the alpha-, mu- and pi-class isozymes) were among the first to be structurally characterized. The first structure determined for any GST was of the porcine pi-class enzyme (pGSTP1-1) (Reinemer et al. 1991) This isozyme contains the indicative features of members of the cytosolic

GST superfamily and serves as a point of comparison with other isozymes: an N-terminal thioredoxin-like domain (with  $\beta\alpha\beta\alpha\beta\alpha$  topology) and a C-terminal domain composed of  $\alpha$ -helices (Figure 1a). The determination of the structure of rat (rGSTK1-1) (Ladner et al. 2004) and human (hGSTK1-1) (Li et al. 2005) mitochondrial GSTs revealed striking topological similarities and differences with respect to the cytosolic GSTs. The kappa-class enzymes have a thioredoxin-like domain, but with a DsbA-like  $\alpha$ -helical domain inserted between helix- $\alpha 2$  and strand- $\beta 3$  (Figure 1b). The mPGES-2 protein represents a special case: the structure contains the topological features of the cytosolic GSTs but with additional structural elements (Yamada et al. 2005) (Figure 1c). In cytosolic GSTs, cytosolic GST-like proteins and mitochondrial GSTs, the binding-site for GSH (the “G-site”) is formed by the thioredoxin-like domain. A fundamentally conserved interaction found in all classes of cytosolic GSTs and in mitochondrial GSTs is a *cis*-proline residue (found at the N-terminal end of strand  $\beta 3$ ) that forms hydrogen-bond interactions with the backbone amine group of the GSH-cysteinyl moiety. The region containing helix  $\alpha 2$  shows the greatest variability among the various classes. This secondary structure element contains residues that interact with the glycine-residue of GSH. Global analysis of sequence and structural similarity of the cytosolic GSTs reveal two major subgroups (Atkinson and Babbitt 2009). These subgroups can be classified as Y-GSTs, which utilize a tyrosine residue to activate glutathione, and the S/C-GSTs that utilize serine (or in the beta- and omega-class GST, cysteine) in their interaction with GSH. In both the Y- and S/C-GSTs (and mitochondrial GSTs), the catalytic GSH-activating residue occurs in a “catalytic loop” following the first  $\beta$ -strand in the thioredoxin-like domain. In humans, the Y-GSTs include alpha, mu, pi, sigma-class isozymes, with all other classes of GST falling into the S/C-GST subfamily. Where a



catalytic tyrosine or serine is present, the hydroxyl groups' of these residues donate a hydrogen bond to the GSH sulfhydryl, promoting the formation of thiolate anion. The importance of this hydroxyl group has been demonstrated in several mutagenesis studies. For example, the hGSTP1-1 Y7F mutant shows severely attenuated activity (Kong et al. 1992a) and that the Y7 hydroxyl group acts to lower the  $pK_a$  of GSH sulfhydryl group by 2.4 pH units (Kong et al. 1992b). Similar studies have confirmed the importance of serine in S/C-type GSTs [e.g. in human theta-class (Tan et al. 1996)], although the function of catalytic-loop cysteine residue in beta-class GSTs is not as clear (Allocati et al. 2000). The kappa-class isozymes resemble the theta-class GSTs in that they utilize a GSH-activating serine residue. The omega-class GSTs utilize a catalytic cysteine residue and, like the beta-class isozymes, can form a mixed disulfide with GSH. They are thus able to catalyze reductive reactions (Board et al. 2000). In this way the omega-class GSTs are similar to the glutaredoxins which also form mixed disulfides with GSH. The intriguing CLICs have retained the ability to bind GSH and, like glutaredoxins beta- and omega-class GSTs, can form mixed disulfides with it. To date, no GST-related activities have been detected in CLICs. The location of the binding site for hydrophobic co-substrates (the "H-site") was first revealed in the crystal structure of the human pi-class GST, hGSTP1-1 (Reinemer et al. 1992), which was co-crystallized with S-hexyl glutathione. It is adjacent to the G-site and in the cytosolic GSTs is formed in a cleft between the N- and C-terminal domains, both of which may contribute residues to its formation. The H-site varies greatly in shape and chemical character between classes. Several examples are described below.

#### *MAPEG*

Recent significant achievements in the understanding of MAPEG protein structure are the determination of MGST1 from rat at 3.2/4.0 Å resolution by electron crystallography (Holm et al. 2006), the X-ray crystal structures of human LTC<sub>4</sub> synthase (LTC<sub>4</sub>-S) in its apo and GSH-complexed forms at 2.00 and 2.15 Å (Martinez Molina et al. 2007) and 3.3 Å resolution (Ago et al. 2007) (Figure 2). Of clinical relevance was the structure of 5-lipoxygenase-activating protein (FLAP) with MK-591 and an iodinated derivative at 4.25 and 4.0 Å resolution (Ferguson et al. 2007). MK-591 is an inhibitor of leukotriene B<sub>4</sub>, C<sub>4</sub>, D<sub>4</sub> and E<sub>4</sub> synthesis and is in trials for the treatment of asthma. All of these are unrelated to the cytosolic- and mitochondrial GSTs, and belong to subgroup-I of the MAPEG family. All the MAPEG proteins are left handed four- $\alpha$ -helix bundles (TM1 to TM4) (Figure 1d) assembled into trimers, with the helical bundles oriented orthogonally in the plane of the membrane (Figure 1d, 2a). The overall topology of MGST1 and LTC<sub>4</sub>-S is strikingly similar to subunit I of ba3-cytochrome *c* oxidase. There are three GSH-binding sites per trimer, located proximal to the cytoplasmic side of the predicted membrane-spanning regions. The MGST1 structure suggested that GSH was bound in an extended conformation, similar to that found in the c- and mitochondrial GSTs, however in the higher resolution LTC<sub>4</sub>-S structures, it is in a V-shaped conformation with its thiol group oriented toward the membrane where it can presumably interact with its membrane-bound substrate. The binding site itself appeared to be formed between two monomers and the cytoplasmic extension of helix-TM2 contributes the most residues (Figure 2c). The key GSH-binding residues in LTC<sub>4</sub>-S are R51, N55, E58, Y59, Y93, Y97 and R104 and from the adjacent monomer, R30 and Q53. Residue R104 appears to play a key role in GSH-activation: the positive charge on the guanadinium group, and its direct interaction with the GSH-thiolate would be expected to promote the pK<sub>a</sub>

shift necessary for deprotonation (Figure 2c). The structures of FLAP with MK-591 and homolog show that the inhibitors bind in pocket adjacent to the GSH-binding site and inside the membrane-spanning regions.

### **GSTs in detoxification**

Because of their role in detoxification, both human and pathogen GSTs have been the subject of considerable study. For example, polyaromatic hydrocarbons (PAHs, an important class of environmental pollutant) oxidized by CYP450s are substrates for multiple classes of GST. Another example from oncology concerns human tumors that express raised levels of pi-class GST. hGSTP1 expression is correlated with resistance to chemotherapeutics such as chlorambucil, cyclophosphamide, BCNU (N,N'-bis(2-chloroethyl)-N-nitrosourea), and cisplatin (Laborde 2010). The structures of several GSTs and their interactions with substrates and inhibitors have been determined.

Early efforts to determine the structures of GSTs focused on inhibitors used to enhance the efficacy of chemotherapeutic agents that they detoxify. Ethacrynic acid, [2,3-dichloro-4-(2-methylenebutyryl)phenoxy]acetic acid (EA), is a well-characterized inhibitor of alpha-, mu- and pi-class GSTs. Originally used as a diuretic, EA is probably the most extensively studied GST inhibitors, and was used in clinical trial with chlorambucil for the treatment of drug-resistant chronic lymphocytic leukemia (Tew 1994). EA is both an inhibitor and substrate of hGSTP1-1 (Figure 3a). The crystal structures of both hGSTA1-1 (Cameron et al. 1995) and hGSTP1-1 (Oakley et al. 1997c) in complex with the diuretic drug EA and its GSH-conjugate (GS-EA) have been determined (Figure 3b, c, d ,e). These studies were among the

first to demonstrate that the H-sites of the pi- and alpha-class GSTs bind similar compounds distinctly. The alpha-class isozyme has an additional  $\alpha$ -helix ( $\alpha 9$ ) at its C-terminus that occludes the H-site (Figure 3d, e). When hGSTA1-1 binds EA, both the H- and G-sites are at least partially occupied, blocking entry of GSH (Figure 3). However, in the hGSTP1-1 complex, EA is bound entirely in the H-site, albeit in a manner not productive for conjugation with GSH. The structure of hGSTP1-1 with bound GS-EA demonstrated that rearrangement of the substrate in the active site was essential for catalysis in that isozyme. The structure also indicates that the Y108 hydroxyl (in the H-site) participates in conjugation through the donation of a hydrogen-bond to the ketone moiety of EA, stabilizing the Michael addition reaction (LoBello et al. 1997) (Figure 3c). The structure of hGSTA1-1 in complex with GS-EA shows that the EA-moiety is partially disordered, with two orientations can be observed. Curiously, a second orientation of the EA-moiety in the hGSTP1-1 GS-EA complex was observed (Oakley et al. 1997a). Taken together, the results suggested that GSTs have considerable versatility in the binding of ligands in the H-site.

The metabolism of PAHs represent an important detoxification function of GSTs. Phenanthrene epoxide (PE), a product of cytochrome P450 oxidation of phenanthrene (Figure 4a) can be conjugated to GSH by rat mu-class isozymes rGSTM1-1 and M2-2. The rGSTM2-2 isozyme is stereospecific in the addition of GSH to the phenanthrene epoxide, giving exclusively (9*S*,10*S*)-9-(S-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene (9*S*,10*S*-GSP). In contrast, the rGSTM1-1 subunit is less efficient and exhibits little stereoselectivity in this reaction and gives rise to both 9*S*,10*S*-GSP and 9*R*,10*R*-GSP diastereoisomers (Boehlert and Armstrong 1984). The structure of rGSTM1-1 was determined with both diastereoisomers (Ji et al. 1994)

(Figure 4b). In the complex with the (9*S*,10*S*)-diastereoisomer, the 10'-hydroxyl group of the ligand is hydrogen-bonded to Y115-OH suggesting that this residue could act as an electrophile to stabilize the transition state for the epoxide ring-opening reaction.

Benzo[*a*]pyrene is a PAH whose metabolites are mutagenic and highly carcinogenic. The carcinogenic metabolites are stereoisomers of 7,8-dihydroxy-9,10-oxo-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE). The (+)-*anti*-isomer, [(+)-*anti*-BPDE] is the more potent carcinogen (Slaga et al. 1979) (Figure 5a). Allelic variants of hGSTP1 have different rates of conjugating activity toward this compound: the activity of hGSTP1\*B-1\*B (containing the I to V substitution at position 104) with (+)-*anti*-BPDE is > 3-fold higher than that of the common I104-containing (1\*A) isoform. The structures of these two isoforms with the conjugation product GS-BPD (Ji et al. 1999) (Figure 5a) reveal that residue 104 positions the reaction product (Figure 5b, c) and suggest that substrate binding in the hGSTP1\*B H-site is more favorable than in the hGSTP1\*A H-sites. The catalytic advantages afforded by a valine residue at position 104 are as follows: a favorable Y7-OH to GS-BPD sulfur-atom distance (3.2 Å), a hydrogen-bond between R13 guanadinium-NH and the 8'-OH group of the BPD moiety, and the positioning of a water molecules in the H-site that mediates a hydrogen-bond between Y108-OH and 9'-OH group (Figure 5c). The absence of these interactions in the hGSTP1-1 complex suggests that polarization of the BPD-OH groups assists in attack of GSH thiolate during catalysis. Analogies appear in the differences in GSH-conjugating activity of the mouse alpha-class GSTs mitochondrial GSTA1-1 and mitochondrial GSTA2-2 toward (+)-*anti*-BPDE. The GSH-conjugating activity of the mitochondrial GSTA1-1 isozyme toward (+)-*anti*-BPDE is

approximately 3-fold higher than that of mitochondrial GSTA2-2. The complex of GS-BPD with mitochondrial GSTA1-1 (Gu et al. 2000) (Figure 5d) and mitochondrial GSTA2-2 (Gu et al. 2003) (Figure 5e) facilitate a structural explanation for these differences. Three H-site residues at positions 207, 216 and 221 appear to play a critical role. Residue I221 and M207 in allow for positioning of the BPD moiety so that the GS-sulfur is 3.25 Å from the Y7-OH group. A guanadinium-NH group of residue R216 donates a H-bond to the 8'-OH group in an analogous fashion to R13 in the pi-class GST. Finally, the glycyl-NH moiety of GSH donated a H-bond to the 7'-OH of BPD that may represent substrate assistance in catalysis. In the mitochondrial GSTA2-2 complex, the enzyme donates no H-bonds to the hydroxyl oxygen atoms of the BPD-moiety and the glutathione sulfur is rotated away from the Y7-OH group (distance = 5.2 Å). The positioning of the aromatic ring by F221 and L207 side chains do not allow rotation of R216 into the H-site to interact with the BPD-OH groups.

In addition to the conjugation of PAH derivatives, certain of the alpha-class GSTs have activity toward products of lipid peroxidation. Structural studies on human (Bruns et al. 1999) and murine (Xiao et al. 1999) GSTA4-4 have sought to explain the Michael addition activity of that isozyme with lipid peroxidation products such as 4-hydroxynon-2-enal (HNE). The reaction is intriguing because of the catalytic efficiency is high, yet lacks stereoselectivity (Figure 6a). The reaction is complicated by the fact the GS-HNE conjugate undergoes reversible cyclization. The cyclized form of GS-HNE was observed in a complex with mGSTA4-4 (Xiao et al. 1999) (figure 6b). The mechanism remained unclear until recently, when the crystal structures of hGSTA4-4 and a chimeric variant of hGSTA1-1 were solved in complex with an isosteric analogue of GS-HNE (Balogh et al. 2010), where the aldehyde

moiety was replaced with an alcohol group (Figure 6c). The structures show that the active site residue R15 can interact with the 4'-OH group of either HNE enantiomer and assisting catalysis through the polarization of the group through the donation of a H-bond. A tyrosine residue peculiar to the A4-4 isozyme, Y212, contributes a hydrogen bond to the aldehyde oxygen atom during Michael addition and thus stabilizes the enolate reaction intermediate. The absence of this tyrosine residue in other alpha-class GSTs explains the enhanced activity of the A4-4 isozyme. The catalytic loop-Y9 residue appears to act in the usual manner, activating GSH for reaction with the  $\alpha,\beta$ -unsaturated moiety.

The role of GSTs in chemotherapy resistance is of clinical significance. Increased levels of pi-class GST have been observed in several tumor cell lines that are resistant to a range of anti-cancer drugs, for example, chlorambucil (Tew 1994). The anticancer drug chlorambucil is a known substrate for hGSTP1-1 and the complex with chlorambucil and the chlorambucil-GSH conjugate have been reported (Parker et al. 2008). Chlorambucil was shown to bind in the H-site and substrate must undergo re-arrangement in the active site for conjugation with GSH to occur. This phenomenon was observed with EA as noted above. With the aforementioned role of hGSTP1-1 in chemotherapy resistance, efforts to find specific inhibitors were undertaken. Evaluation of GST inhibitors with specifically toward hGSTP1-1 over other classes lead to the discovery of TLK117 [ $\gamma$ -glutamyl-(S-benzyl)cysteinyl-D-phenylglycine] (Figure 7a), the most selective hGSTP1-1 inhibitor reported to date (Lyttle et al. 1994). The compound has a  $K_i$  of 0.4  $\mu$ M with hGSTP1-1, but over 50-fold greater with alpha- and mu-class isozymes. The structure of the TLK117-hGSTP1-1 complex reveals the basis for the selectivity of the compound for pi-class

isozymes (Oakley et al. 1997b). The D-phenylglycine moiety of the inhibitor contacts hydrophobic residues whilst maintaining the multiple hydrogen-bond interactions that occur between GSH and the G-site. The substitution of the glycyl moiety in GSH by D-phenylglycine in TLK-117 would result in steric clashes in the alpha and mu- class enzymes, which maintain intimate contacts with the glycyl residue of GSH (Figure 7b). In the clinic, TLK117 is administered as a diethyl-ester prodrug, TLK199. While the potentiating effects of TLK199 in tumor cells were insufficient to warrant clinical trials, TLK199 was shown to act as a small-molecule myeloproliferative agent (Laborde 2010). These activities may be due to the disruption of GSTP1-1 with c-Jun N-terminal kinase, resulting in the modulation of the kinase pathways that affect cell proliferation.

### **GSTs as ligandins**

Ligandin, originally identified as a cytoplasmic protein that bound various compounds including bilirubin, heme, xenobiotics, steroids and azo-dyes was identified with GST activity in 1974 (Habig et al.). There is considerable interest in the “ligandin” function of GSTs. This activity contributes to intracellular sequestration and transport of xenobiotics and hormones. The location of this site appears to vary depending on the chemical nature of the ligand and GST-class. Of great interest was the determination of the structure of a mu-class GST from *Schistosoma japonica* with praziquantel (McTigue et al. 1995), an anthelmintic effective against flatworms. The drug was shown to bind in the dimer interface (Figure 8a, b). This was the first structure in which ligand binding outside of the G- and H-sites was demonstrated in a cytosolic GST. Several structures of the human pi-class GST feature binding of the buffer-substances MES or HEPES at a site adjacent to W28, located at the N-terminal end of



strand  $\beta 2$  (Ji et al. 1997; Oakley et al. 1997b; Prade et al. 1997) (Figure 8c, d). Later studies demonstrated binding of ligands in various binding sites: Sulfasalazine (a sulfa-drug), cibacron blue (a sulfonated triazine dye) and bromosulphophthalein (used in liver function tests) bind in the H-site (Oakley et al. 1999). Binding of ligands in the dimer interface is also possible in alpha-class GSTs: affinity labeling of rat GSTA3-3 has revealed a ligandin site within the cleft between the two subunits (Vargo and Colman 2001). In addition to binding D<sup>5</sup>-androstene-3,17-dione in the H-site (see below), rGSTA3-3 binds displays high affinity for 17 $\beta$ -estradiol-3,17-disulfate. Finally, the structure of a sigma-class GST (from squid) in complex with S-(3-iodobenzyl)-GSH demonstrates non-substrate binding in the dimer interface in addition to the expected mode of binding in the G-and H-site (Ji et al. 1996). Taken together the data suggest that observations of ligand-binding interactions in one class cannot easily be extrapolated to other classes, or indeed between chemically distinct ligands binding to a particular GST isozyme.

### **GSTs in hormone biosynthesis**

Among the GSTs are enzymes that are involved in the biosynthesis of leukotrienes, prostaglandins, and steroid hormones. The mammalian sigma-class GSTs catalyze the conversion of PGH<sub>2</sub> to PGD<sub>2</sub>, whereas mPGES' catalyses the conversion of PGH<sub>2</sub> to PGE<sub>2</sub> (relevant structures are shown in Figure 9). Both utilize GSH as a cofactor in their respective reactions. As noted above mPGES-2 contains a cytosolic GST-like domain. mPGES-1, however, is a member of MAPEG subgroup IV (Hayes et al. 2005). Some members of the MAPEG family catalyze the formation of LTC<sub>4</sub> and PGE<sub>2</sub> (Hayes et al. 2005). An alpha-class GST isozyme has been demonstrated to catalyze 3-keto- $\Delta^5$ -steroids to 3-keto- $\Delta^4$ -steroid isomers (Johansson and Mannervik

2001). Atomic-resolution structures of some relevant cytosolic GSTs and the non-membrane-bound fragment of GST-like mPGES-2 are available, and progress has been made into the understanding of structures of the integral membrane MAPEG enzymes, however there is currently a paucity of structural data on how the hormone co-substrates bind, and catalytic mechanisms have been inferred based on modeling. There are nevertheless several structures of these enzymes with inhibitors of clinical relevance.

### *Leukotrienes*

The hematopoietic prostaglandin D synthase, (HPDS, a sigma-class GST), catalyses the isomerization of PGH<sub>2</sub> to PGD<sub>2</sub>, a mediator of allergy and inflammation. PGH<sub>2</sub> is an unstable peroxide-containing derivative of arachidonic acid. The structure of the rat HPDS (Kanaoka et al. 1997) displayed features in common with the alpha, mu and pi class enzymes (such as a GSH-activating tyrosine residue) but had a unique H-site structure for binding its largely aliphatic substrate. Divalent cations decrease the  $K_m$  for PGH<sub>2</sub> and GSH, increasing PGDS activity. The structure of the human isozyme (Inoue et al. 2003) revealed structural details of Ca<sup>2+</sup> and Mg<sup>2+</sup>-mediated activation. These ions are bound in the dimer interface *via* octahedrally coordinated water molecules, which are in turn bound to acidic residues clustered around the central two-fold axis (Inoue et al. 2003) (Figure 10). One acidic residue, D96 accepts a hydrogen-bond from R14, which binds GSH. The role of HPDS in inflammation makes it an attractive drug target for inhibition. While no structure of HPDS with a substrate or reaction product exists, several enzyme/inhibitor complex structures have been determined. A fragment-based screening approach lead to the identification of an inhibitor, phenyl-5-(1H-pyrazol-3-yl)-1,3-thiazole with an IC<sub>50</sub> of 21 nM, one of

the most potent inhibitors of HPDS to date (Figure 10c) (Hohwy et al. 2008). A recent study demonstrated the binding of nocodazole, an anti-neoplastic agent ( $IC_{50} \approx 65$  mM) (Weber et al. 2010) (Figure 10b, c)

It is worth noting briefly the proposed catalytic mechanism for HPDS: a hydrogen bond from the Y8-OH to GSH-thiol was proposed to result in the formation of thiolate anion, which engages in nucleophilic attack on the oxygen atom of  $PGH_2$ . A  $GS^-$  anion in solution then engages in nucleophilic attack on the C11-hydrogen atom of the GSH- $PGH_2$  adduct, causing O-S bond cleavage and the formation of the C11 carbonyl. A hydrogen atom transferred to the C9-hydroxyanion from GSH completes the formation of the product (Figure 10d) (Kanaoka et al. 1997).

The mPGES-2 enzyme (Figure 1c) is another target for inhibition due to its  $PGE_2$ -forming activity.  $PGE_2$  is a regulator of biological activities including smooth muscle dilation and contraction (Jakobsson et al. 1999b). The N-terminal domain of mPGES-2 contains a CPXC active-site motif resembling that found in thioredoxins (Yamada et al. 2005). Noteworthy is the fact that mPGES-2 does not require GSH for its catalytic activity, although the catalytic rate can be increased from two- to fourfold by DTT, dihydrolipoic acid, GSH or other thiol compounds (Tanikawa et al. 2002). The structure has been determined in complex with GSH and indomethacin (IMN), a non-steroidal anti-inflammatory drug and cyclooxygenase inhibitor. Indomethacin is a weak inhibitor of mPGES-2 ( $IC_{50} \approx 1$  mM) (Yamada et al. 2005) (Figure 11a, b). The mechanism is unclear but appears to involve the first cysteine of the CPXC motif (C110). Although not closely related to HPDS described above, modeling of the substrate in mPGES-2 suggested intriguing mechanistic parallels. When mPGES-2

and HPGDS are superimposed, the C110-SH group of the former overlaps with the GSH-thiol of the latter, suggesting that the thiols could perform equivalent functions during catalysis. In an update to their previous structural studies on mPGES-2, Yamada and Takusagawa (2007) determined the structure of this protein as a complex with GSH and heme. Heme lies in the same site as indomethacin (Figure 11c). The affinity of mPGES-2 for heme is high ( $K_d = 0.53$  mM) but the physiological role is unclear. It was suggested that the GSH-heme complex-bound enzyme catalyzes the formation of 12(*S*)-hydroxy-5(*Z*),8(*E*),10(*E*)-heptadecatrienoic acid (HHT) and malondialdehyde from PGH<sub>2</sub>, however, later biochemical analysis using more sensitive assays suggested that HHT production is due to the non-enzyme catalyzed reaction of PGH<sub>2</sub> with heme (Watanabe et al. 2008). Thus the functional importance of heme-binding remains an open question.

### *Steroids*

Despite having a similar amino acid sequence compared to other human alpha-class GSTs, hGSTA3-3 is unique in having high steroid double bond isomerase activity for the conversion of  $\Delta^5$ -androstene-3,17-dione to  $\Delta^4$ -androstene-3,17-dione (Gu et al. 2004). While the structure of this GST has been determined with GSH, the complex with a co-substrate has not. However, based on a model of  $\Delta^5$ -androstene-3,17-dione in the H-site of hGSTA3-3 (Figure 12a), a mechanism was proposed in which the abstraction by GS<sup>-</sup> of a hydrogen ion from the 4'-carbon of the steroid is followed by isomerization and the transfer of a hydrogen ion to the 6'-carbon. The formation of thiolate anion is promoted by the active-site tyrosine (Y9). In this scheme, GSH acts as a general base rather than substrate. The activity of hGSTA3-3 in this reaction was rationalized in terms of two active site residues (G12 and A208) that differ with

respect to other alpha-class isozymes. The equivalent residues in hGSTA1-1 (A12 and M208) would clash with the substrate and prevent binding. The hGSTA3-3 mutants G12A and A208M showed significantly attenuated activity (Gu et al. 2004).

### **A GST in Tyrosine catabolism**

The zeta-class GSTs, also known as maleylacetoacetate isomerase (MAAI) catalyzes the isomerization of maleylacetoacetate to fumarylacetoacetate (Figure 13a) with GSH as a co-factor, and many other reactions. Its MAAI activity makes it a key enzyme in the catabolism of phenylalanine and tyrosine. The other activities of the zeta-class GSTs include oxygenation, dehalogenation, peroxidation, and transferase activities. Deficiencies in enzymes along the tyrosine degradation pathway lead to diseases including phenylketonuria, alkaptonuria, and fatally, hereditary tyrosinemia type I. Of clinical interest is the dichloroacetate (DCA) transforming activity of the enzyme: DCA is used clinically for the treatment of congenital lactic acidosis, its mode of action being the promotion the metabolism of lactic acid via activation of the pyruvate dehydrogenase complex (Stacpoole et al. 1998) (see the Anders and Board review in this issue). The structure of human MAAI (hGSTZ1-1) was reported at 1.9 Å resolution in complex with glutathione and a sulfate ion (Polekhina et al. 2001) (Figure 13b). While the structure has characteristic features of the cytosolic GST fold, it was not clear which if any of the catalytic loop residues function in an analogous fashion to the archetypal GSH-activating residues of other classes. Subsequent investigation of the catalytic activity of point-mutants toward model substrate 2-bromo-3-(4-nitrophenyl)propionic acid suggested that of residues S14, S15 and C16, only S14 was essential for activity. Based on the increased  $K_m$  for GSH of the C16A mutant, C16 was proposed to position GSH in the active site (Board et al. 2003). This

observation was somewhat puzzling because S14 does not appear to interact directly with GSH (Figure 13a). Further insight into catalysis in this class of GST comes from the structure of maleyl pyruvate isomerase (MPI) from *Ralstonia sp.* U2, a zeta-class GST homolog that shares 40% sequence identity with the human isozyme (Marsh et al. 2008). Like the human zeta-class enzyme, it is an isomerase utilizing GSH as a cofactor. This enzyme functions in the pathway for degradation of naphthalene *via* gentisate that enables the bacterium to utilize polycyclic aromatic hydrocarbons as its sole carbon source. The substrate is very similar to that of the human isozyme (Figure 13a). The structure was determined as a complex with dicarboxyethyl-GSH (an analogue of the proposed reaction intermediate) (Figure 13c, d). The structure suggests that the GSH thiolate attacks the substrate in the 2'-position, with a carboxylate buried at the base of the active site cleft, forming hydrogen bonds with H104-N $\epsilon$ 2 and crucially, the backbone-NH group of G10 (equivalent to S15 in the human isozyme). This interaction helps explain the importance of the preceding residue (S14 in the human isozyme) for catalysis despite the orientation of the side-chain away from the G-site. An arginine residue (R176) was proposed to stabilize the formation of enolate oxygen at the 4'-carbon during the Michael addition of GSH (Marsh et al. 2008). The arginine residue is highly conserved (R175 in hGSTZ1-1) and this mechanism could also operate in other zeta-class GSTs including hGSTZ1-1 (Figure 13e).

## Conclusions

From a structural perspective, GSTs are the best-characterized enzyme in detoxification: structural data covers GSTs of multiple phyla, classes and functions. The binding of relevant toxins, drugs, and other ligands to mammalian cytosolic

GSTs are well characterized. Efforts to uncover the structural secrets of the integral-membrane MAPEG family of proteins have, in the past five years, yielded fruit. Nevertheless, the structural basis for the activity of several classes of GSTs of relevance to human health remains mysterious.

Bacterial beta-class GSTs such as that of *Proteus mirabilis* are proposed to contribute to antibiotic resistance through binding of tetracyclines and rifamycin (Perito et al. 1996). The structure of PmGSTB1-1 has been determined (Rossjohn et al. 1998b), but its binding activities are yet to be structurally characterized. Nevertheless, substrate binding in one bacterial GST has been described: the structure of BphK from *Burkholderia xenovorans* LB400 in complex with (2Z,4E)-2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid (HOPDA), a product of PCB breakdown, has been described (Tocheva et al. 2006).

Further research is needed to characterize substrate binding and catalysis in mitochondrial kappa-class GSTs. To date, structures of the rat (Ladner et al. 2004) and human (Li et al. 2005) kappa-class GSTs have been determined in the presence of glutathione or its analogues but not with a relevant co-substrate. Nevertheless, understanding of co-substrate binding in this class has been aided by investigations into a bacterial kappa class GST from *Pseudomonas putida* known as 2-hydroxychromene-2-carboxylic acid (HCCA) isomerase (Thompson et al. 2007). While the sequence identity of HCCA with rGSTK is only 22%, the overall topology is highly similar (the RMSD of the superposed structures is 1.9 Å over 180 Cα atoms).

The characterization of parasite GSTs will be a fertile area of research in the future, and may have potential as targets for inhibition. For example, the malarial parasite *Plasmodium falciparum* possesses one cytosolic GST isoenzyme (PfGST), the structure of which was determined (Fritz-Wolf et al. 2003). The sequence and structure of PfGST shows that cannot easily be assigned to any extant GST class. The activity of PfGST is increased in chloroquine-resistant cells, and has been shown to act as a ligandin for parasitotoxic hemozoin. PfGST represents a promising target for antimalarial drug development. In another example, the structure of a novel class of GST (nu) from the model hookworm nematode *Heligmosomoides polygyrus* (HpGSTN2-2) has been solved (Schuller et al. 2005). The GST activity of nematodes is up-regulated during chronic infection of their hosts.

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## Figure legends

**Figure 1.** Topology and structural representations of (a) cytosolic GST (b) mitochondrial GST (c) mPGES and (d) MAPEG. The thioredoxin-like domains in (a), (b) and (c) are represented in green. The C-terminal domain particular to cytosolic GST and mPGES-2 is in cyan (a, c). Additional structural elements of mPGES-2 (c) are shown in yellow and magenta. The DsbA-like insertion in mitochondrial GSTs (b) is shown in cyan. The approximate location of the membrane around membrane-spanning portion of MPGES-2 is shown by horizontal bars. In all cartoon representations, GSH is represented in stick-form.



**Figure 2.** Cartoon diagrams of (a) MGST1 (Holm et al. 2006) and (b) LTC<sub>4</sub>-S (Martinez Molina et al. 2007) as viewed from the cytoplasmic face. The monomers in each structure are colored red, green and cyan. GSH is shown in stick form. In (c) the GSH binding site of LTC<sub>4</sub>-S is shown. Residues contributed from adjacent monomers in green or cyan. The critical GSH-activating R104 is labeled in red. Helices are labeled TM1 or TM4.

**Figure 3.** (a) hGSTP1-1 catalyzed conjugation of EA with GSH. The structures of hGSTP1-1 (b, c) and hGSTA1-1 (d, e) are shown as complexes with EA (b, d) and the EA-GSH conjugate (c, e). Proteins are represented in cartoon form with ligands and key residues drawn in stick form. The domains are colored as for Figure 1a. The distinct C-terminal extension of hGSTA1 is colored red. hGSTA1-1 in panel E contains the R15K mutation.

**Figure 4.** (a) Conjugation reaction of PE with GSH. (b) Structures of rGSTM1-1 in complex with 9*S*,10*S*-GSP (white) or 9*R*,10*R*-GSP (yellow). rGSTM1-1 is drawn in cartoon form, using the color convention of Figure 1a.

**Figure 5.** (a) Conjugation of BPDE with GSH. The structures of (b) hGSTP1-1 and (c) allelic variant P1\*B (I104V) in complex with GS-BPD in cartoon form with key residues in stick form. The substituted residue is labeled in red. The complexes of BPD-GS with mGSTA1-1 (d) and mGSTA2-2 (e) are illustrated with the reaction product and key residues in stick form. In all cases the protein backbone is

represented in cartoon from, colored according to the convention in Figure 1 and Figure 3.

**Figure 6.** (a) Conjugation of GSH with HNE. (b) structure of mGSTA1-1 in complexed with cyclized GS-HNE. (c) Structure of hGSTA1-1 in complex with isosteric GS-HNE analog glutathionyl-1,4-dihydroxynonanol. Ligands and critical residues are shown in stick form. The protein backbone is represented in cartoon from, colored according to the convention in Figure 1 and Figure 3.

**Figure 7.** (a) Chemical structure of TLK117. (b) hGSTP1-1 (green), hGSTA1-1 (red) and hGSTM2-2 (tan) are shown superposed. TLK117 and selected residues are shown in stick form.

**Figure 8.** (a) Cartoon representation of *Schistosoma japonica* GST dimer with praziquantel (b) bound in the dimer interface. (c) Human pi-class GST with MES (d) bound. The color convention of figure 1 is used.

**Figure 9.** Structures of PGH<sub>2</sub>, PGE<sub>2</sub> and PGD<sub>2</sub>.

**Figure 10.** (a) Binding of Mg<sup>2+</sup> in the dimer interface of HPDS. Residues and water molecules linking GSH to Mg<sup>2+</sup> are shown in stick form. (b) The active site of HPDS with nocodazole (yellow carbon atoms) or phenyl-5-(1H-pyrazol-3-yl)-1,3-thiazole (black carbon atoms) bound, and (c) their chemical structures. (d) Proposed mechanism of GSH-assisted conversion of PGH<sub>2</sub> to PGD<sub>2</sub>.

**Figure 11.** (a) Active-site of mPGES-2 (cartoon form) with indomethacin (b) bound. (c) Active-site of mPGES-2 with GSH-heme bound. The ligands are drawn with black carbon atoms and the contacting residues with yellow carbon atoms. The color convention of figure 1c is used here.

**Figure 12.** (a) Model of hGSTA3-3 as a complex with  $\Delta^5$ -androstene-3,17-dione ( $\Delta^5$ AD) and GSH, shown in stick form. The color convention from Figure 3 is used. (b) proposed mechanism of isomerization.

**Figure 13.** (a) Isomerization of maleylacetoacetate ( $R = CH_2COO^-$ ) and maleylpyruvate ( $R = COO^-$ ) to fumarylacetoacetate and fumarylpyruvate. Structure hGSTZ1-1 (b) and MPI (c) in the vicinity of the active-site. Ligands and surrounding residues are in stick form. All ligands are drawn with black carbon atoms. The color convention of figure 1 is used for the backbone cartoon. Residue R176 (c) occupies two discrete conformations. (d) The structure of dicarboxyethyl-GSH. (e) Proposed reaction mechanism of hGSTZ1-1 and MPI.

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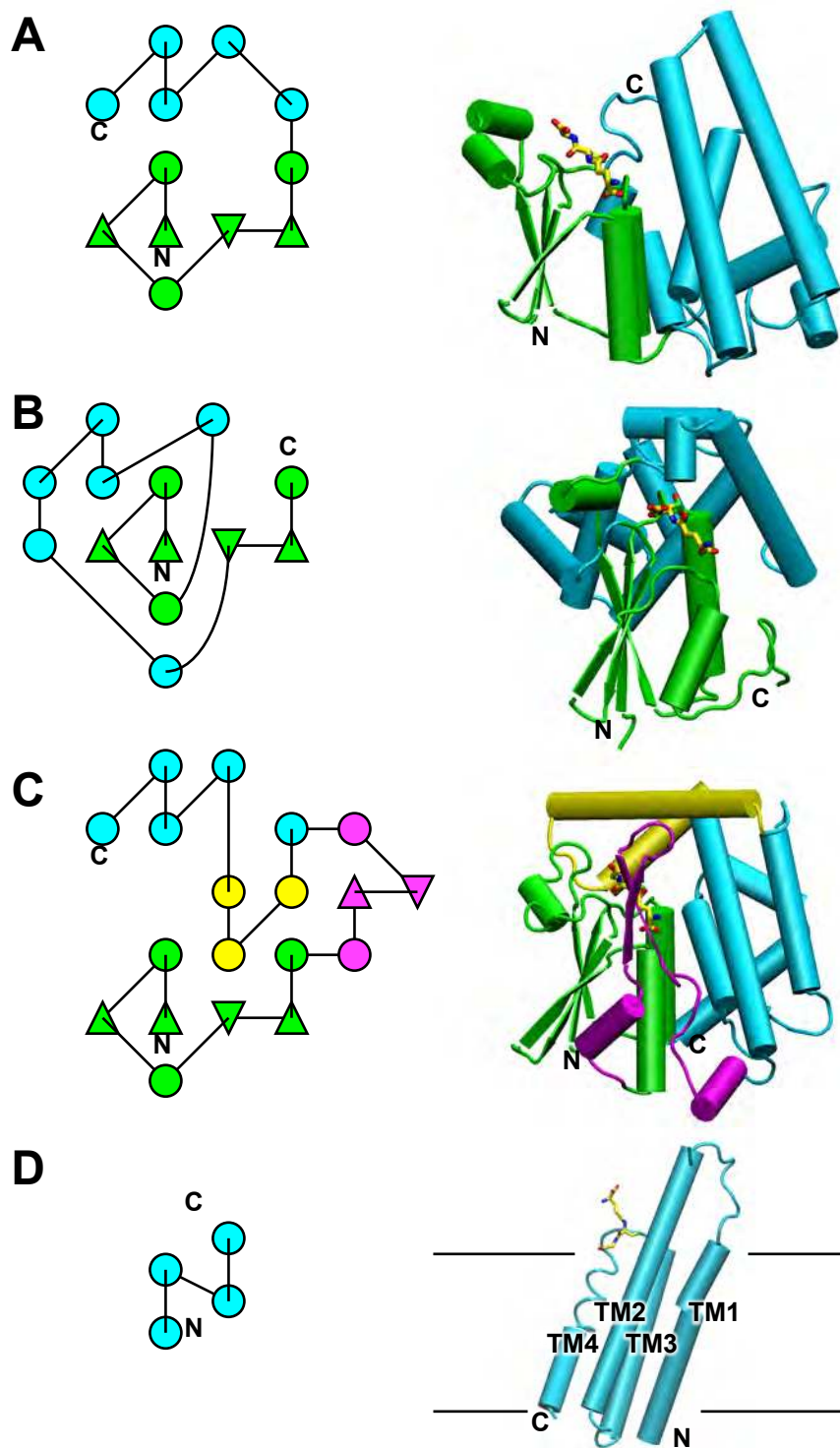
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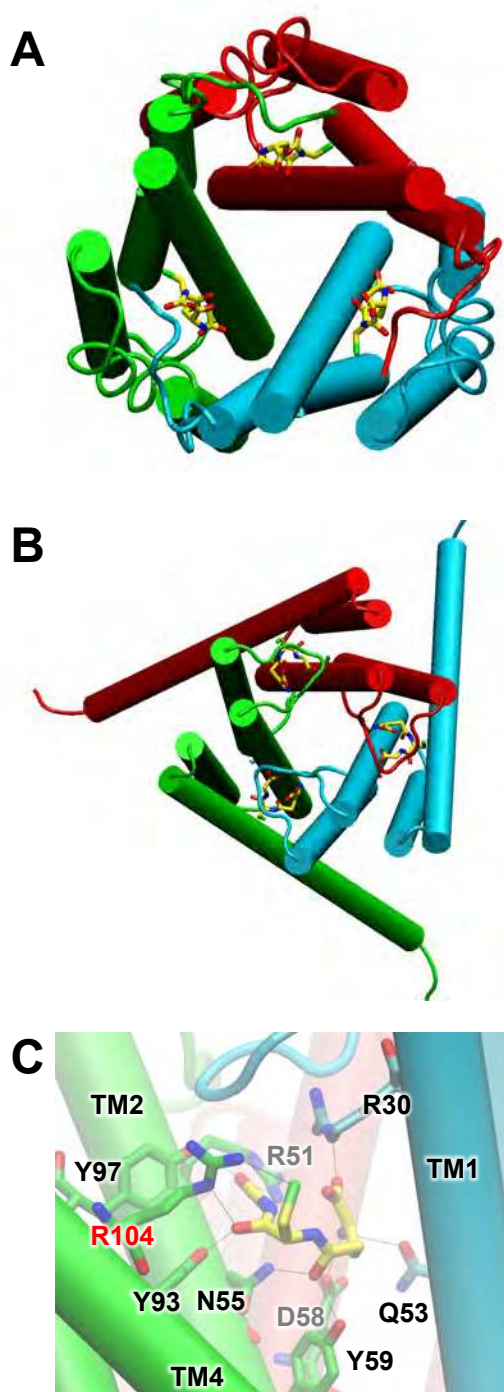


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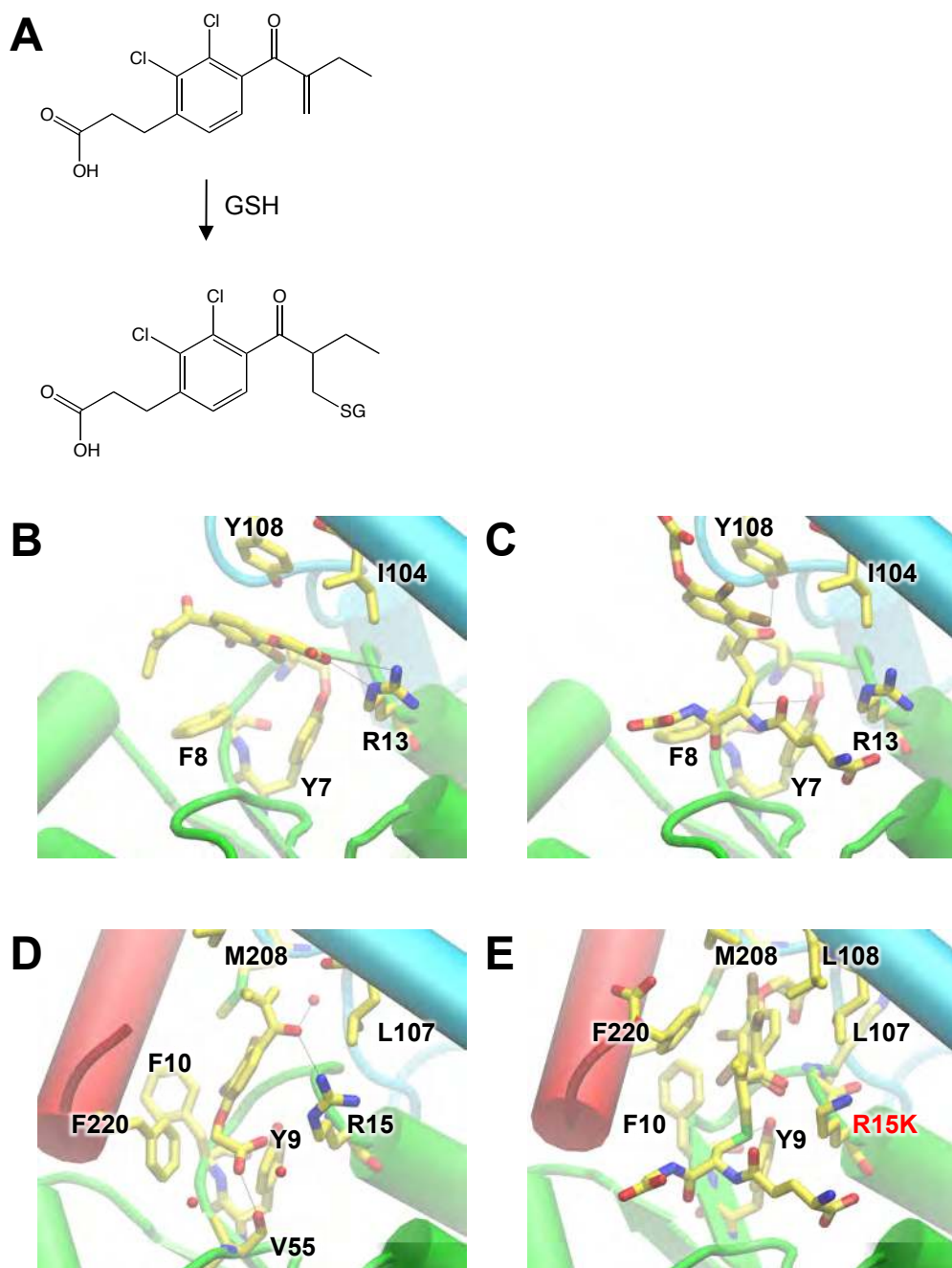




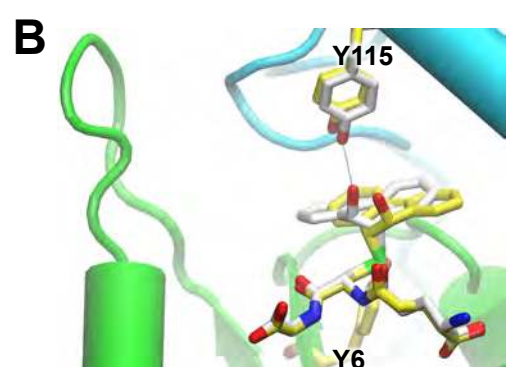
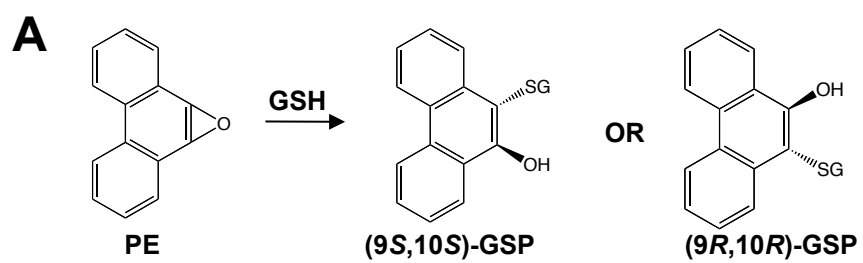
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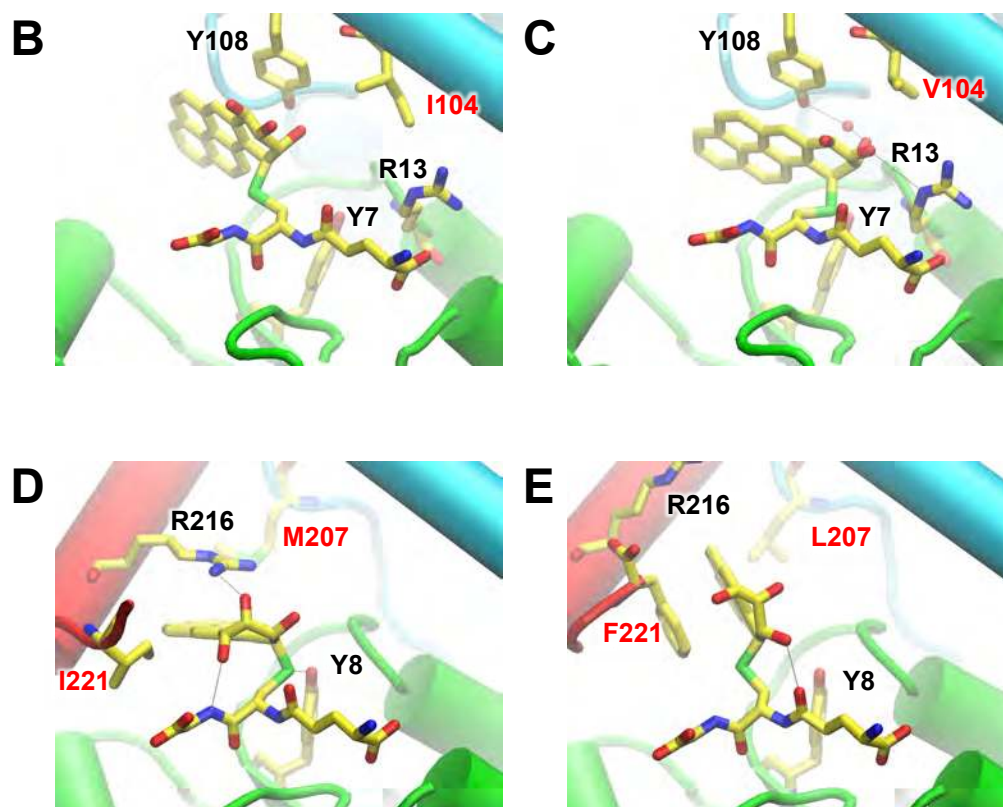
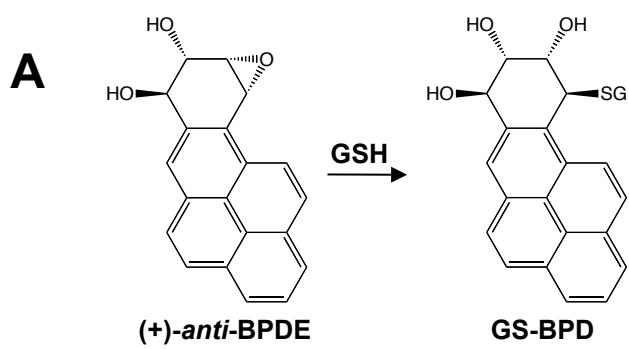
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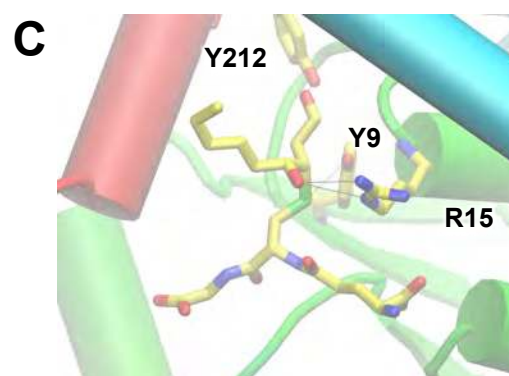
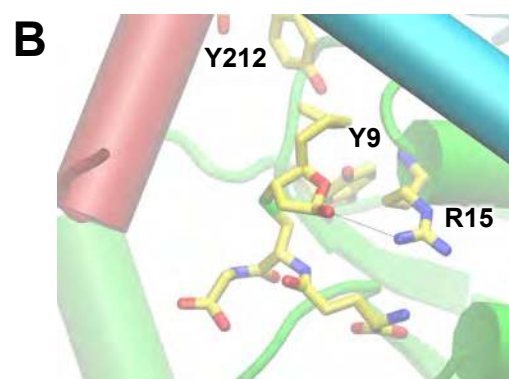
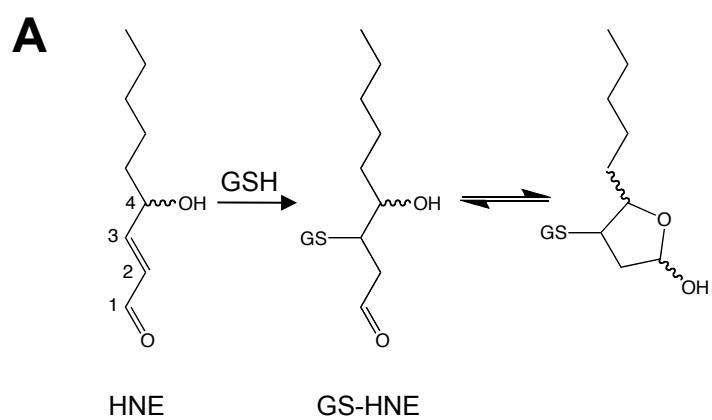
**Figure 3**



**Figure 4**

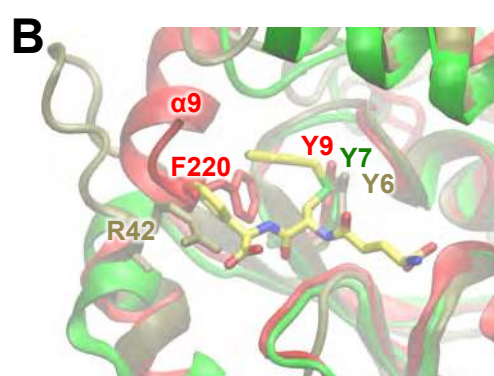
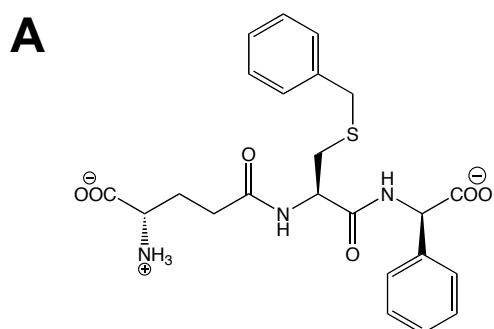


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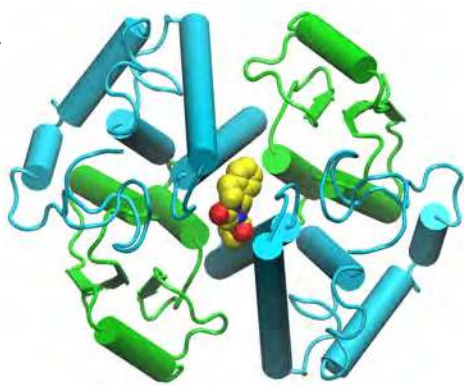
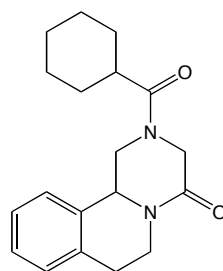
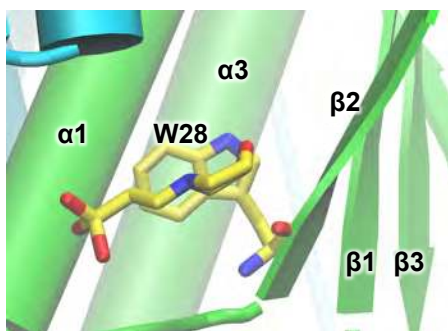
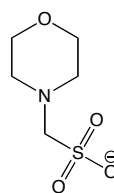


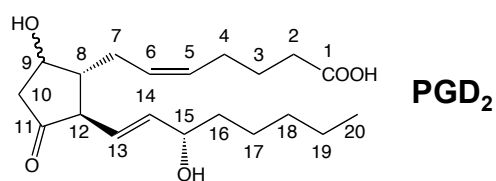
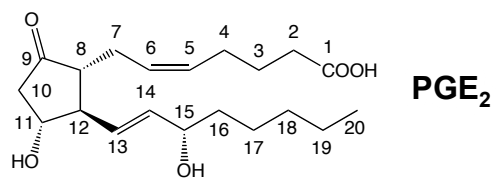
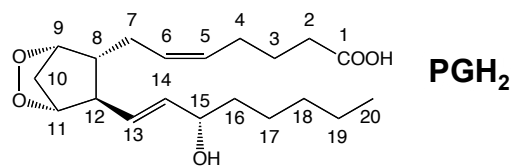
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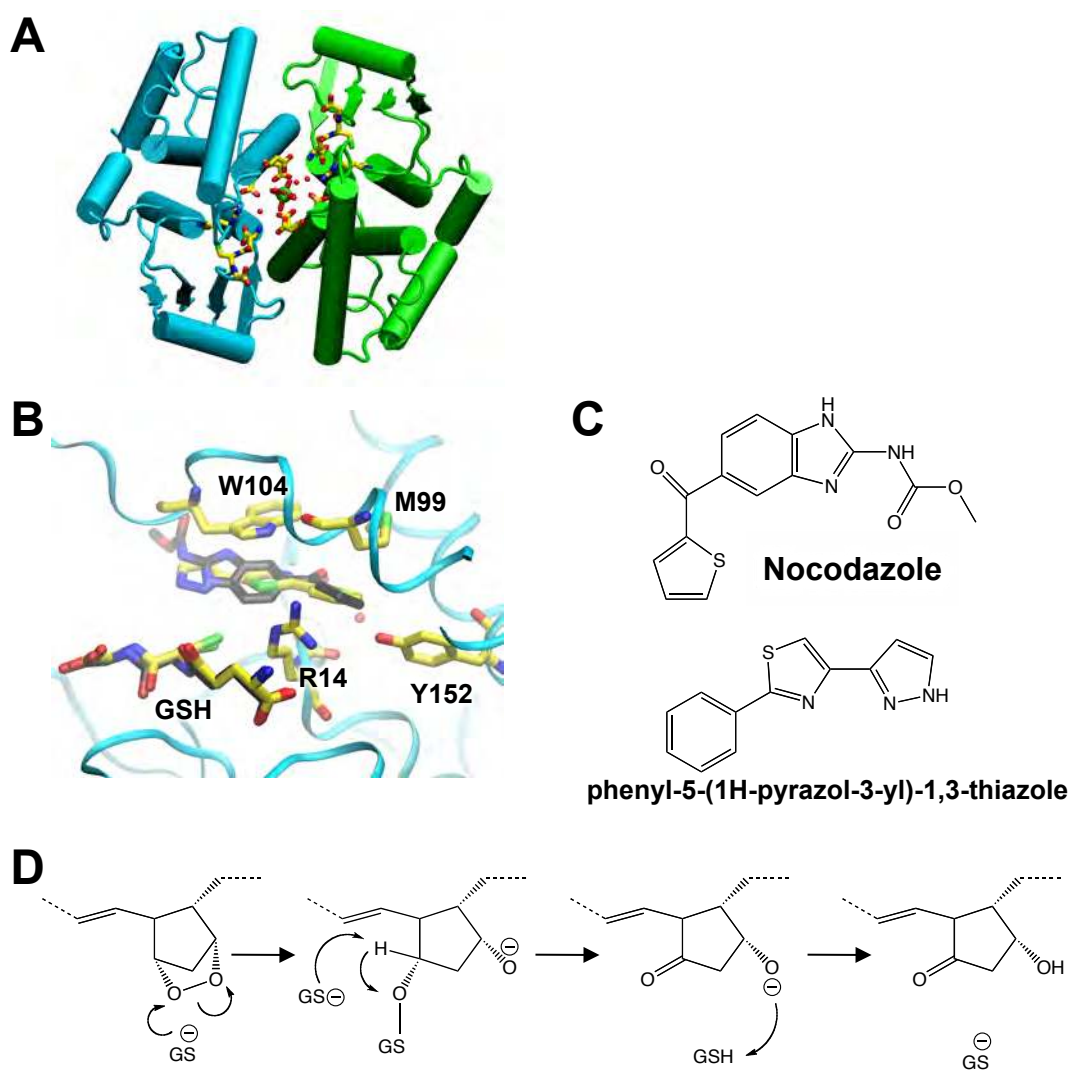


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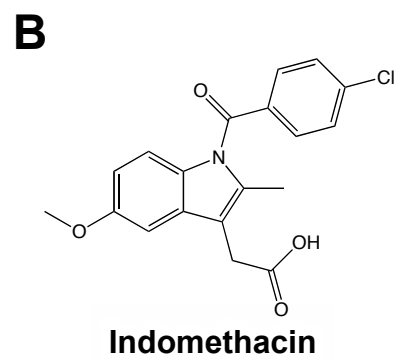
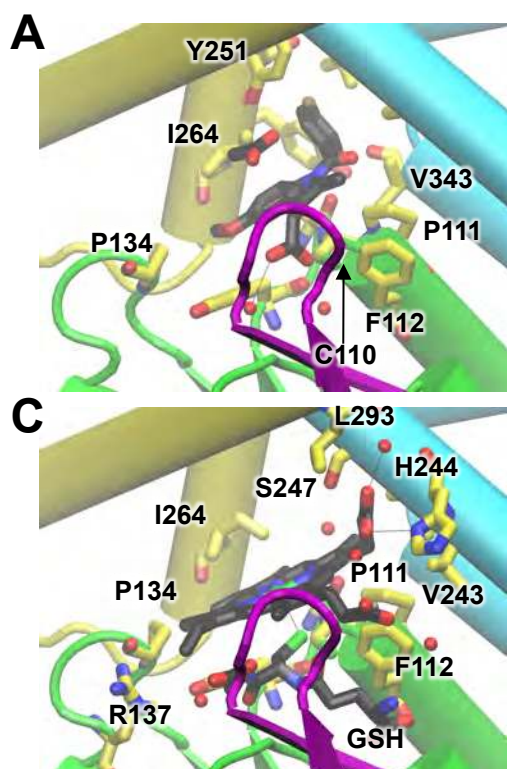
**A****B****C****D****Figure 8**



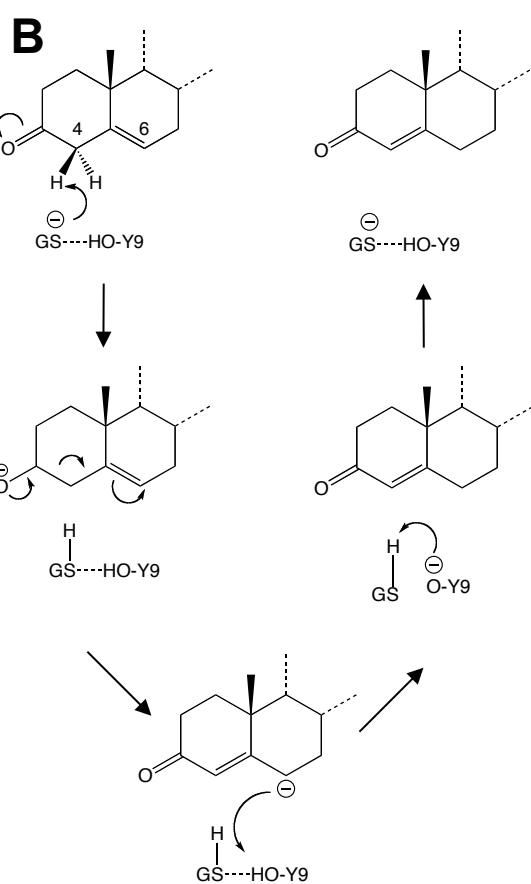
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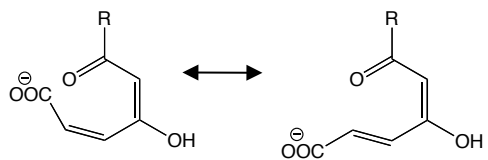
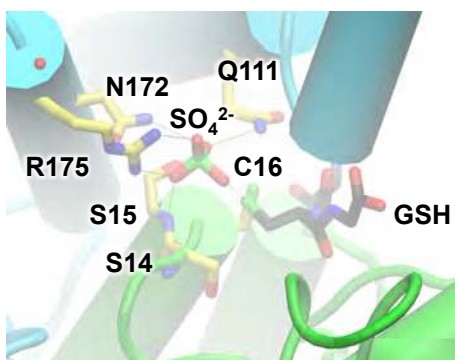
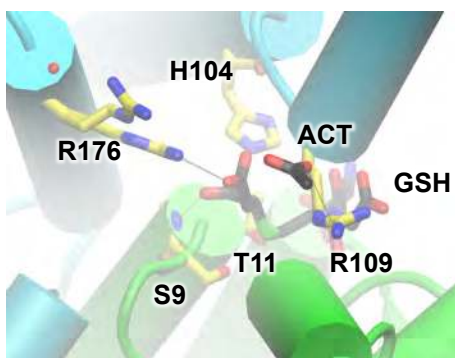
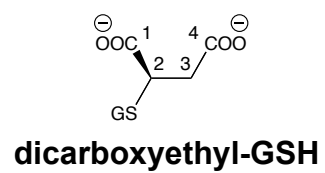
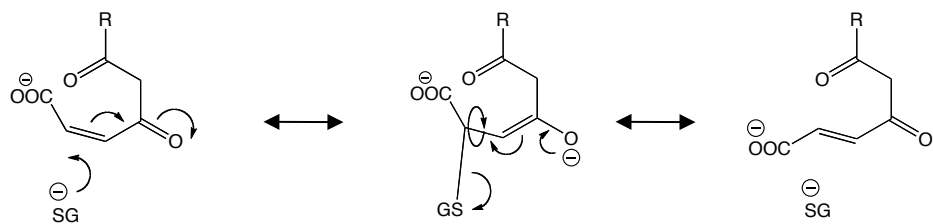
**Figure 10**



**Figure 11**



### Figure 12

**A****B****C****D****E****Figure 13**