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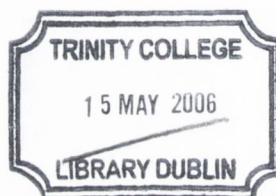
# **STUDIES ON THE THREE CYSTEINES OF MOUSE GLUTATHIONE S-TRANSFERASE P1**

A dissertation submitted to Trinity College Dublin, for the degree of Doctor of  
Philosophy

By

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August 2005

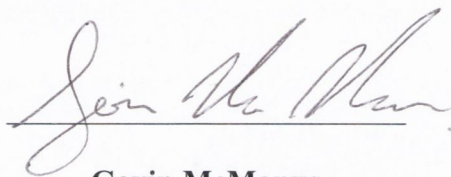




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**Gavin McManus**

## **Acknowledgements**

I would like to thank my supervisor Dr. Tim Mantle, without whom this thesis would never have been finished. Thank you Tim for all your patience and help and for trying to pass on your unlimited curiosity in all things scientific, although you still haven't convinced me on the bird-watching thing. Also a big thanks to everybody in the lab, to Orla and Aisling who have moved on to bigger and better things and to the lads Ed and Seamus for all those Wednesday nights and for all the suffering together as we wrote up. Also to all the other guys in the lab, Berti, Jerrard, Coilin, Tania, Vinny and Catriona.

Also I would like to say a big thank you to Sinead Boyce for proof-reading this thesis: between you and Tim you made it look like English isn't my second language; to Georgia for help with some of the pictures and for being a good friend, and everybody in the department who has made it a good place to pass the time, including Jude and Dan who have since gone to pastures new.

A very big thank you to my parents for putting up with me all these years and not disowning me for being the eternal student.

And sure to anybody who knows me, cheers.

## ABSTRACT

The present study has identified the “alkaline thiol” of mGSTP1 originally described by Phillips and Mantle (1993) as cysteine-169. In addition cysteine-47 is confirmed as the reactive “neutral” pK thiol. Cysteine-14 in the C14\* mutant is unreactive at neutral pH and only slowly reacts under alkaline conditions. Several key experiments however suggest that Cys-14 can form a disulphide with Cys-47 and this may occur under conditions when the basic dimeric structure is maintained. A comparison of the extent of reaction of DTNB with GSTP1 forms with only Cys-14 or only Cys-47 sum to a significantly lower value than obtained when both Cys-14 and Cys-47 are present. A simple mechanism to explain this has been presented where an initial mixed disulphide between NTB and Cys-47 destabilises the  $\alpha$ 2-loop allowing Cys-14 to attack the modified Cys-47 forming a stable disulphide between Cys-14 and Cys-47 and releasing NTB. Whether this thioredoxin like half reaction has any functional significance is presently unclear, however it should be noted that treatment of C169A with relatively low concentrations of  $\text{H}_2\text{O}_2$  resulted in the formation of the Cys-14/Cys-47 disulphide as revealed by a species running with an apparent mobility of 20kDa on SDS/PAGE in the absence of any mercaptoethanol.

Advantage has been taken of the availability of the cysteine to alanine mutants of GSTP1 to develop a reagent to study glutathionylation reactions *in vitro*. A simple procedure has been developed to isolate GSSN, a novel reagent that clearly glutathionylates the reactive thiol (Cys-47) of GSTP1. This reaction is monitored by the release of the yellow reporter NTB which allows the reaction to be followed spectrophotometrically. The synthesis of  $\text{G}^{35}\text{SSN}$  has allowed the fate of the glutathione moiety to be followed more easily. The additional thiols of native mGSTP1 (C14 and C169) clearly displace the glutathione, bound initially to Cys-47, as GSSG. In preliminary experiments it was argued that this displacement reaction may have been due to the less reactive but accessible cysteine-169. However the retention of radiolabelled glutathione by the C169A mutant was only slightly improved when compared with the wild type. Stable glutathionylation was achieved with C47\*, where only the low pK thiol of Cys-47 is present. Intriguingly the C14A mutant also retained approximately 0.5 mole glutathione per mole of subunit, further evidence that Cys-14 can react with Cys-47 when it is modified to a mixed disulphide. The implications of this work for our understanding of the role of glutathionylation in signalling are discussed.

Kinetic studies of all the cysteine to alanine mutants have revealed two surprising features. Firstly, cooperative binding of glutathione is the normal behaviour, suggesting that the wild type may have been selected for suppressing this intrinsic tendency. Secondly two mutants exhibit apparently ping-pong kinetics. This may be explained if the proton release step from GSH is irreversible, although other explanations are possible. Several lines of evidence suggest that a crucial water molecule, W0, may be the first acceptor of the GSH proton. How the release of this solvent molecule may become essentially irreversible for the C14A and C169A mutants will require further work.



**ABBREVIATIONS USED IN THE TEXT.**

|                 |                                           |
|-----------------|-------------------------------------------|
| A <sub>nm</sub> | Absorbance                                |
| ARE             | Antioxidant response element              |
| ASK             | Apoptosis signal-regulated kinase         |
| bp              | Base pairs                                |
| BSA             | Bovine serum albumin                      |
| bZIP            | Basic leucine zipper transcription factor |
| CDNB            | 1-chloro-2,4-dinitrobenzene               |
| cpm             | Counts per minute                         |
| CTABr           | cetyltrimethylammonium bromide            |
| CuOOH           | Cumene hydroperoxide                      |
| Da              | Dalton                                    |
| DMSO            | Dimethyl sulfoxide                        |
| DNTB            | 5,5'-Dithiobis-(2-nitrobenzoic acid)      |
| dNTP            | Deoxyribonucleotide triphosphate          |
| dpm             | Disintegrations per minute                |
| DPNG            | Dinitrophenylglutathione                  |
| DTT             | Dithiothreitol                            |
| EDTA            | Ethylenediaminetetraacetic acid           |
| GAPDH           | Glyceraldehyde-3-phosphate dehydrogenase  |
| GSH             | Glutathione                               |
| GSSG            | Oxidized glutathione                      |
| GSSN            | Glutathionyl-S-S-nitrobenzoate            |
| GST             | Glutathione-S-transferase                 |
| HNE             | 4-Hydroxy-2-nonenal                       |
| HO-1            | Haem oxygenase-1                          |
| IPTG            | Isopropylthio-β-D-galactose               |
| JNK             | Jun N-terminal kinase                     |
| kDa             | KiloDalton                                |
| KGF             | Keratinocyte growth factor                |

|               |                                                                                |
|---------------|--------------------------------------------------------------------------------|
| MAPEG         | Membrane associated proteins involved in eicosanoid and glutathione metabolism |
| MAPK          | Mitogen-activated protein kinase                                               |
| MARE          | Maf recognition element                                                        |
| NADPH         | Nicotinamide adenine dinucleotide phosphate                                    |
| Nrf2          | NF-E2 related factor 2                                                         |
| NTB           | Nitrothiobenzoate                                                              |
| ori           | Origin of replication                                                          |
| PBS           | Phosphate buffered saline                                                      |
| PCR           | Polymerase chain reaction                                                      |
| PKA           | cAMP-dependent protein kinase                                                  |
| PKC           | Protein kinase C                                                               |
| PPAR $\gamma$ | Peroxisomal proliferator-activated receptor $\gamma$                           |
| PTEN          | Phosphatase and tensin homologue deleted on chromosome 10                      |
| PTP           | Protein tyrosine phosphatase                                                   |
| ROS           | Reactive oxygen species                                                        |
| rpm           | Revolutions per minute                                                         |
| SDS           | Sodium dodecyl sulfate                                                         |
| SDS-PAGE      | SDS-polyacrylamide gel electrophoresis                                         |
| Spn-GS        | <i>S</i> -( <i>p</i> -nitrobenzyl)-glutathione                                 |
| TAE           | Tris-acetate/EDTA electrophoresis buffer                                       |
| TBE           | Tris-borate/EDTA electrophoresis buffer                                        |
| TBS           | Tris buffered saline                                                           |
| TCA           | Trichloroacetic acid                                                           |
| TE            | Tris/EDTA buffer                                                               |
| TEMED         | <i>N,N,N',N'</i> -tetramethylenediamide                                        |
| Tris          | 2-amino-2-(hydroxymethyl)-propane-1,3-diol                                     |
| Trx           | Thioredoxin                                                                    |
| TTBS          | Tris/Tween 20 buffered saline                                                  |
| Tween 20      | polyoxyethylene (20) sorbitan monolaurate                                      |
| XBP           | Xanthophyll binding protein                                                    |

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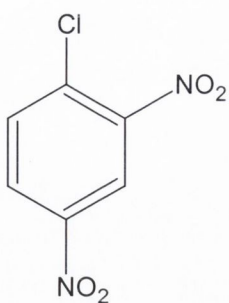
# Chapter 1



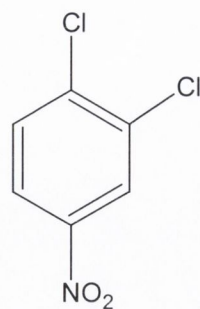
## 1.1 Introduction to Glutathione S-transferases

### 1.1.1 Functions of GST

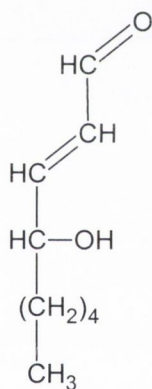
Glutathione *S*-transferases (GSTs; EC 2.5.1.18) are soluble proteins with typical molecular masses of approximately 50 kDa. They are composed of two polypeptide subunits, Mr 22-28 kDa and may be homo or heterodimers. Classically, GSTs catalyse the transfer of the tripeptide glutathione ( $\gamma$ -glutamyl-cysteinyl-glycine; GSH) to a co-substrate (R-X) containing a reactive electrophilic centre to form a polar S-glutathionylated reaction product (R-SG) and liberating HX. As will become clear later in this thesis, the fact that a proton is a reaction product is often forgotten. The glutathione conjugates are generally less reactive than their parent compounds. Many toxic xenobiotics are strongly lipophilic, therefore, glutathione conjugation makes them more polar, preventing them from becoming incorporated into membranes and marks them for export by a membrane-bound GS-X pump (Ishikawa *et al.*, 1992). These enzymes were first discovered in animals in the 1960s as a result of their importance in the metabolism and detoxification of drugs. GSTs are specific for GSH as one of their substrates, although homoglutathione is the preferred substrate in legumes for certain reactions with a stress-induced GST, glycine max1 (GSTGm1) (Skipsey *et al.*, 1997). However they have a more general range of second substrates. GSTs conjugate compounds containing an electrophilic centre, which can be a carbon, nitrogen or sulfur atom (Jakoby *et al.*, 1978). The most widely used GST electrophilic substrate for assaying GST activity is 1-chloro-2,4-dinitrobenzene (CDNB), because the thioether conjugation product has a characteristic absorbance at 340 nm. Other commonly used substrates are shown in Fig. 1.1. Certain isozymes of GST are also



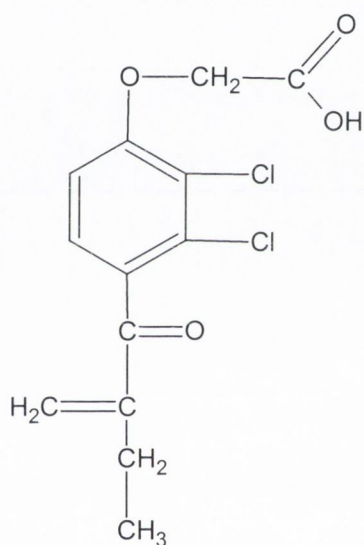
**CDNB**  
(1-chloro-2,4-dinitrobenzene)



**DCNB**  
(1,2-dichloro-4-nitrobenzene)



**4-Hydroxynonenal**



**Ethacrynic acid**

**Figure 1.1 Glutathione *S*-transferase substrates**

Examples of compounds that are electrophilic substrates for the GST family.

efficient at conjugating GSH with 4-hydroxynonenal (HNE), a major genotoxic and cytotoxic  $\alpha,\beta$ -unsaturated aldehyde formed during lipid peroxidation. The reaction of HNE with sulfhydryl groups leads to the formation of thioester adducts that undergo further cyclization, to form hemiacetals (Esterbauer *et al.*, 1991). In addition to their role in catalysing the conjugation of electrophilic substrates to glutathione, these enzymes also carry out a range of other functions including peroxidase and isomerase activity. They are also able to bind (non-catalytically) a wide range of endogenous and exogenous ligands, such as bilirubin and steroids and, in this capacity, were originally described as ligandin (Bhargava *et al.*, 1978). More recently GST P1 and GST M1 have been suggested to modulate the basal activity of jun N terminal kinase (JNK) (Alder *et al.*, 1999) and apoptosis signal-regulated kinase (ASK1) (Cho *et al.*, 2000). Other more physiological roles include the discovery that the zeta class GST is also known as maleylacetoacetate isomerase (MAAI) and is a component of the phenylalanine/tyrosine catabolism pathway (Fernandez-Canon *et al.*, 1999)

### **1.1.2 Classes and tissue distribution of GSTs**

Many molecular forms of GST have been identified, from various organs in different species. The soluble enzymes exist as homo- or heterodimers, which are formed from subunits within a particular class, but not between different classes (Hayes *et al.*, 1979). Mannervik *et al.* (1985) proposed a species-independent classification system. GSTs comprise a complex and widespread enzyme family that has been subdivided into an ever-increasing number of classes based on amino acid/nucleotide sequence, and on immunological, kinetic and structural properties. Nomenclature is based on cDNA sequences. Two evolutionarily separate multigene



families encode the GSTs. One superfamily of GST comprises soluble proteins, whereas the other is composed of membrane-bound transferases. The soluble GSTs found in mammals can be grouped into eight gene families Alpha, Mu, Pi, Sigma, Theta, Zeta, Omega and Kappa (Hayes *et al.*, 2005). In bacteria, insects and plants, further GSTs have been identified and designated Beta, Delta (Enayati *et al.*, 2005), Phi, Tau and Lambda (Dixon *et al.*, 2003). The microsomal transferase family has recently been called MAPEG, membrane associated proteins involved in eicosanoid and glutathione metabolism (Jakobsson *et al.*, 1999). The two superfamilies of GSTs are structurally separate and functionally distinct. Under conditions where there is an increased intracellular concentration of reactive oxygen species (ROS), the soluble transferases act as detoxification enzymes to prevent cytotoxic and genotoxic damage caused by electrophiles generated as breakdown products of macromolecules. However they have been found to have many more functions (Hayes *et al.*, 2005). During oxidative stress, members of the MAPEG family serve to inhibit lipid peroxidation and possibly influence the mobility of arachidonic acid within membranes (Hayes *et al.*, 1999).

Members within any class exhibit similar monomer sizes between 22 and 28 kDa. They share a high amino-acid identity, between 60 and 95% within each class. Homology between members of different classes is less than 30%. Each class is thought to have arisen from a common ancestor gene (Pemble *et al.*, 1992). While the Alpha, Mu and Theta families have diverged greatly, there seems to be an evolutionary constraint on the Pi family. GST genes and proteins from mammalian sources have been especially well characterized, but studies of GSTs from non-mammalian sources have revealed the existence of several new classes and thus



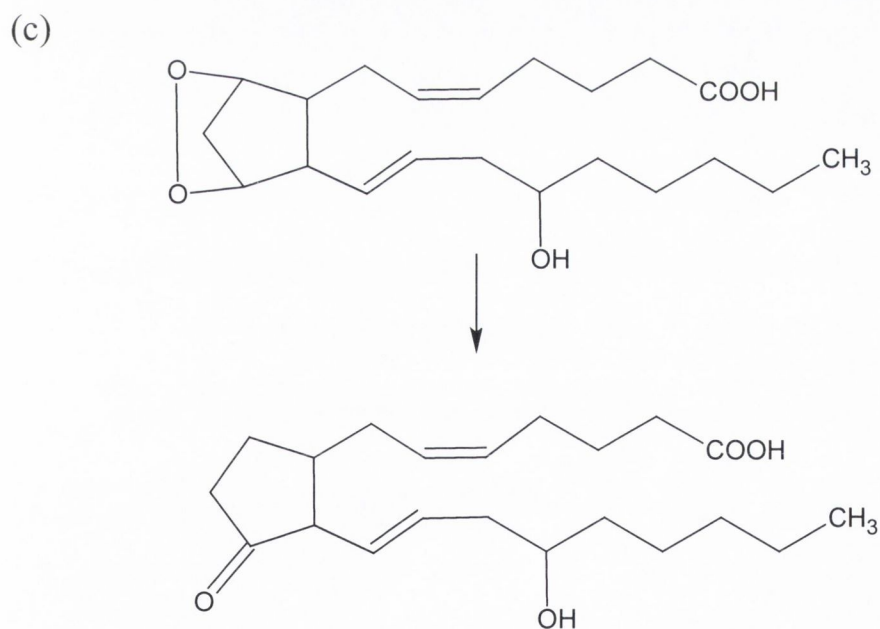
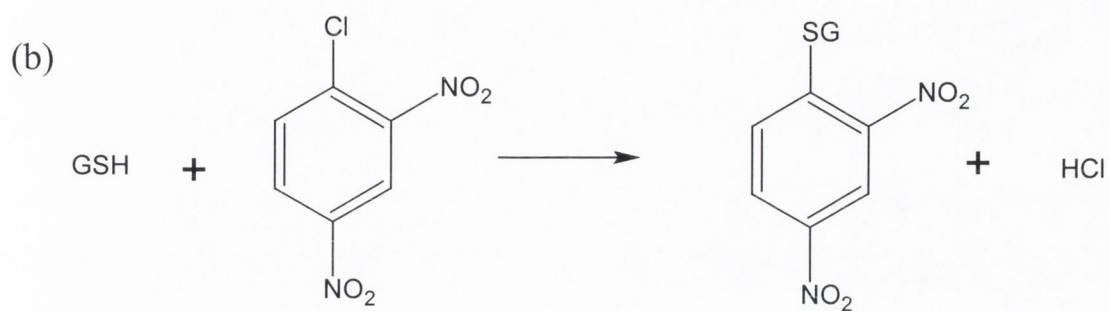
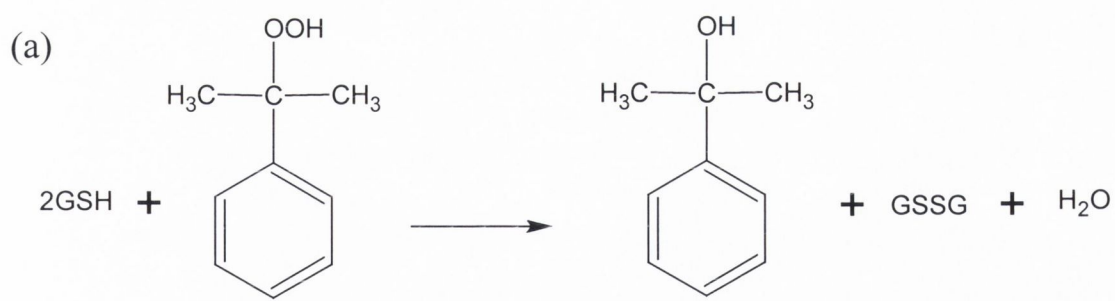
greatly extended our knowledge of the structural and functional diversity of these proteins (Sheehan *et al.*, 2001).

Mouse GST Pi exists as two transcriptionally active genes, namely mGSTP1 and mGSTP2, found in tandem, within 2.5 kb of each other. Both rats and humans possess only one transcriptionally active gene although zebrafish has recently been reported to have two tandem Pi genes (Suzuki *et al.*, 2005). mGSTP1 is found in the brain, heart, kidney, spleen, liver and muscle. In mouse liver, GSTP1 is transcribed at much higher levels than GSTP2 and concomitantly male mice express both GST Pi genes to a greater extent than females. It has been shown that mGSTP1 displays catalytic activity towards a range of substrates up to three orders of magnitude greater than mGSTP2 (Henderson *et al.*, 1998).

There are six differences in amino-acid sequence between the two mGST Pi proteins and it has been established by site-directed mutagenesis that the residues at positions 10, 11 and 104 are responsible for the profound differences in catalytic activity between P1 and P2 (Bammler *et al.*, 1995). Studies suggest that these amino-acid changes affect the binding of both the electrophilic substrate and glutathione to the enzyme.

### **1.1.3 Catalytic mechanism and crystal structures for GSTs**

All families of GSTs contain members that catalyse the conjugation of GSH with CDNB and exhibit glutathione peroxidase activity towards cumene hydroperoxide (CuOOH); (Fig 1.2). The cytosolic GST and MAPEG enzymes catalyse isomerization of various unsaturated compounds (Fig 1.2) and are involved in the synthesis of prostaglandins and leukotrienes (Hayes *et al.*, 1999).



**Figure 1.2 Reactions of glutathione *S*-transferase**

(a) cumene hydroperoxide, (b) 1-chloro-2,4-dinitrobenzene and (c) Prostaglandin  $\text{H}_2$  ( $\text{PGH}_2$ ) conversion to  $\text{PGD}_2$

Ionization of the thiol group is thought to be the first step in catalysis. The increased reactivity of the ionized thiol ( $\text{GS}^-$ ) facilitates attack on the electrophilic centre of the hydrophobic substrate. The pKa of the GSH thiol group is lowered from 9.2 to about 6.5 when bound to the protein, where it is stabilized by a tyrosine side-chain (Graminski *et al.*, 1989) or, in the case of Theta, a serine side-chain.

The most common reaction used to study the kinetic properties of GST is the conjugation of glutathione to CDNB. This reaction is a nucleophilic aromatic substitution and occurs via an unstable Meisenheimer-complex intermediate. The formation of this complex may be monitored using the activated arene 1,3,5-trinitrobenzene, which lacks a good leaving group. It therefore reversibly forms a dead-end Meisenheimer-complex that can be monitored spectrophotometrically (Graminski *et al.*, 1989).

Structures for a large number of cytosolic GSTs have been solved by X-ray crystallography and even though there are big differences in sequences between the different classes, they all have a similar overall structure (Fig 1.3). The first three dimensional structure was determined for the pig lung Pi enzyme (Reinemer *et al.*, 1991). Each monomer has two domains, the N-terminal domain (domain I, residues 1 to 74) contains the bulk of the glutathione binding site, or G site, and has an  $\alpha/\beta$  structure similar to that of thioredoxin. It forms the folding topology  $\beta\alpha\beta\alpha\beta\alpha$ , with the four  $\beta$ -strands forming a regular  $\beta$ -sheet surrounded by the three  $\alpha$ -helices. The C-terminal (domain II, residues 81 to 207), is a helical structure composed of six  $\alpha$  helices (Dirr *et al.*, 1994). The G-site on each monomer was identified using the competitive inhibitor glutathione-sulfonate. The structures of human and mouse GSTP1 have also been solved with a range of inhibitors bound to the active site (Reinemer *et al.*, 1992; Garcia-Saez *et al.*, 1994).



The binding site for the hydrophobic substrate (H-site) was elucidated for the hGSTP1-1 protein using the inhibitor S-hexylglutathione (Reinemer *et al.*, 1992). The substrate binding site was found to be adjacent to the GSH binding site, with the hexyl moiety nestled in a hydrophobic cleft. A structure for mouse GSTP1-1 has been solved with GSH and shows no major conformational changes compared with the protein and glutathione adduct complexes (Parraga *et al.*, 1998). This structure confirms the role of Tyr-7 in stabilizing the thiolate anion ( $\text{GS}^-$ ) by hydrogen-bonding and positioning it in the correct orientation. It also revealed rearrangements in a well-defined network of water molecules in the active site, which included the water molecule W0, identified in the unliganded enzyme (Orozco *et al.*, 1997), which was displaced in the presence of glutathione and W4, which is displaced following the binding of the electrophilic substrate and the formation of the glutathione conjugate. It is suggested that one of these water molecules may play a role in the removal of the proton from glutathione out of the active site. Caccuri *et al.* (1997) showed, using potentiometric experiments, that the proton coming from the bound GSH is released quantitatively into the surrounding solution. This process did not require the presence of a cosubstrate and occurred in the absence of any enzyme turnover.

All GSTs are dimers and there is no evidence for the existence of catalytically active monomers in the living cell. Monomeric forms of GSTP1-1 have been detected in plasma samples (Kura *et al.*, 1996) and have been suggested to play a role in the regulation of JNK signalling (Adler *et al.*, 1999). The only inter-subunit contact to GSH in the active site is by Asp-99. The results of mutational studies performed on this residue in hGSTP1-1 were varied depending on the replacement residue. Replacement of asparagine by leucine in position 99 (D99L) caused almost complete loss of activity, with limited binding of GSH (Manoharan *et al.*, 1992). Kong *et al.*

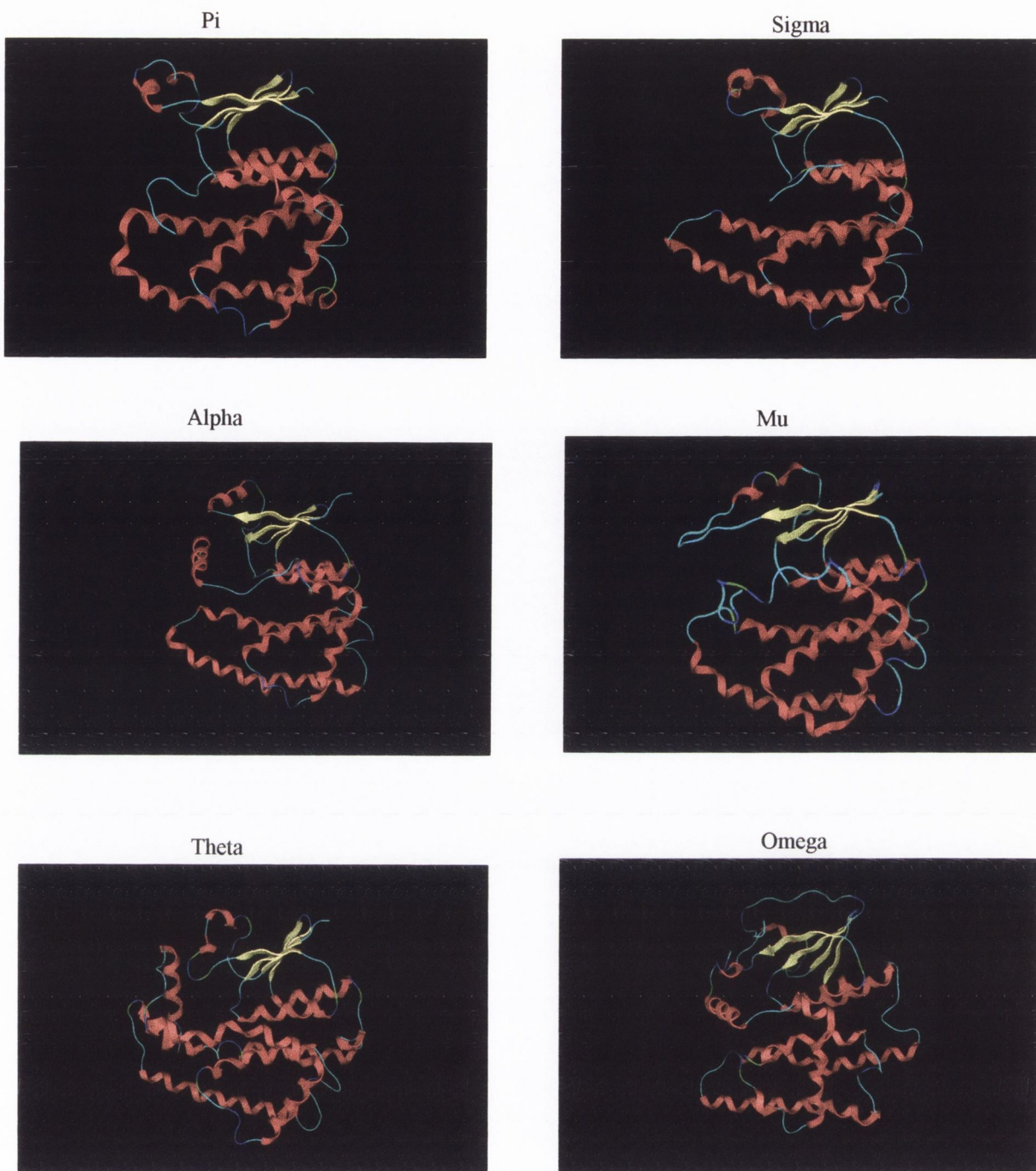


(1993) reported an effect on binding and a smaller impact on catalysis based on studies of the D99A mutant. A monomeric GSTP1-1 that was designed with ten site specific mutations in the subunit interface was found to be structurally stable but catalytically inactive (Abdalla *et al.*, 2002). It is believed that the interface of the dimer plays an important role in the catalytic activity of the enzyme. In the crystal structure of hGSTP1-1, there is a strand of water molecules spaced by distances suited for hydrogen bonding that extends from the active site in one subunit across the two-fold symmetry axis to the active site in the second subunit, through 21 hydrogen bonds (Hegazy *et al.*, 2003). In many of the classes of GST including Alpha, Mu and Pi, there is a V-shaped interface located close to the active site that has a lock and key interaction, where the key is either Phe or Tyr and the lock is formed by five residues in the second subunit. In hGSTP1-1 the lock and key function is obtained by wedging the hydrophobic side-chain of Tyr-50 from one subunit into the lock formed by residues Met-92, Gly-96, Pro-129, Phe-130 and Leu-133 in the other subunit (Reinemer *et al.*, 1992). The helix  $\alpha 2$  loop, which forms the outer wall of the GSH binding site, consists of residues 36-52 and is very mobile. The residues Trp-39, Lys-45, Gln-52 and the the NH group of the Leu-53 backbone amide have H-bond interactions with GSH and contribute to the G-site. In the crystal structure of GSTP1-1 in complex with GSH, Tyr-50 makes 15 van der Waals and hydrogen bonding contacts with the neighbouring subunit, while in the apo-enzyme, there are only eight such contacts (Oakley *et al.*, 1998). The positioning of Tyr-50 inside the lock motif leads to stabilization of the helix  $\alpha 2$  loop. Mutation of Tyr-50 to an alanine reduces the specific activity with CDNB 25,000 fold (Hegazy *et al.*, 2003).

While there is a low level of sequence similarity between certain members of the GST family, they all contain a few conserved regions. The hydrophobic staple motif is found on a substructure formed by helix  $\alpha 6$  and the preceding loop which is known as the GST motif II (Aceto *et al.*, 1997). This motif contains the sequence GXXh(T/S)XXDh (X is any residue and h is a hydrophobic residue) and is maintained in all GSTs known to date (Stenberg *et al.*, 1997). GST motif II is stabilized by a network of eight hydrogen bonds, with half involving a water-mediated contact. GST motif II makes interdomain contacts with helix  $\alpha 1$ , which is an important structural element of the active site (Rossjohn *et al.*, 2000). The highly conserved glycine is located in the bend of the loop preceding helix  $\alpha 6$  and when this is mutated the stability of the protein is affected (Kong *et al.*, 2003).

Example of structures from all classes of cytosolic GSTs have been solved and they all have the canonical GST fold, an N-terminal thioredoxin-like domain, and a C-terminal domain that comprises  $\alpha$ -helices only (Fig 1.3). In the Alpha and Theta class enzymes, the C terminus forms a well defined  $\alpha$ -helical structure that shields the active site and plays an important role in catalysis, through the control of product release. The Mu class enzymes have a characteristic mu-loop, which is located between  $\beta 2$  and  $\alpha 2$  the active site. This results in a deeper active-site cleft than is found in Pi class GSTs. In the case of Omega, there is an extended N terminus of 19 residues when compared to other cytosolic GSTs. This forms a distinct structural unit, which contains a proline-rich segment that, in conjunction with the C terminus forms a distinct structural unit of unknown function (Board *et al.*, 2000). In the case of the Kappa class the enzyme is more closely related to the protein disulfide bond isomerase, dsbA, from *E. coli* than it is to members of the canonical superfamily. The structures of Kappa and the canonical superfamily members indicate that the protein





**Figure 1.3 The common fold of the GST superfamily**

The coordinates were taken for members of the GST superfamily and a monomer of each was shown in a ribbon structure with a helix in red and beta sheet in yellow. Protein data bank codes and references for the structures used are : Alpha, 1gse (Careron *et al.*, 1995); Mu, 1hna (Raghunathan *et al.*, 1994); Omega, 1eem (Board *et al.*, 2000); Pi, 1glp (Garcia-Saez *et al.*, 1994); Sigma, 1gsq (Ji *et al.*, 1995); Theta, 1ljr (Rossjohn *et al.*, 1998).

folds have diverged from a common thioredoxin/glutaredoxin progenitor but that they did so by different mechanisms (Ladner *et al.*, 2004).

## 1.2 Protein thiols

The levels of ROS or oxidative stress can play an important role in deciding the fate of the cell. A toxic insult can lead to membrane dysfunction, DNA damage and inactivation of proteins (Hayes and McLellan 1999). There is increasing interest in the involvement of cysteinyl residues, in proteins as well as glutathione, in response to oxidative stress in the cell. Thiols have been shown to be highly sensitive to oxidative stress. They can be oxidized to sulfenic acid (SOH), and further to sulfinic (SO<sub>2</sub>H) and sulfonic acid (SO<sub>3</sub>H). Sulfenic-acid formation in proteins has been found to occur as a direct result of the reaction of cysteine thiols with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Reactivity of a particular protein thiol towards peroxides and other oxidants is strongly dependent on the ionization state of the thiol (Luo *et al.*, 2005), as thiolate anions are far more nucleophilic than their protonated counterparts.

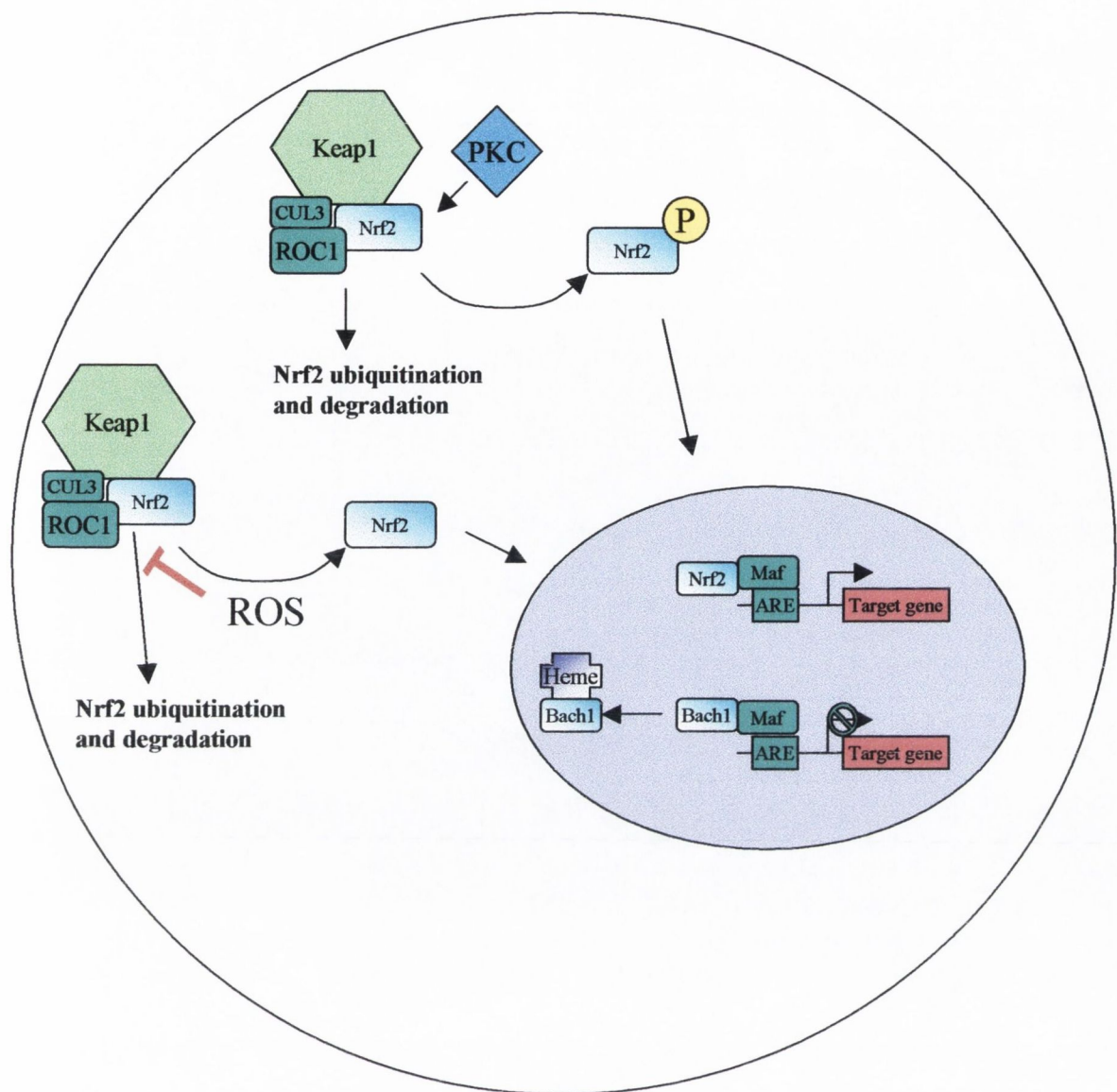
Oxidation of cysteine thiols to sulfenic acid is reversible but further oxidation to sulfinic acid or sulfonic acid has been considered to be biologically irreversible, and in the case of sulfonic acid this is clearly true. There have been reports of reversal of sulfinic acid thiols in yeast where a protein Sxr1 has been shown to be responsible for the ATP-dependent reduction of sulfinic acid on 2-Cys peroxiredoxin (Biteau *et al.*, 2003).

One of the first examples of a regulatory network in response to oxidative stress was the OxyR transcription factor found in *E. coli* (Christman *et al.*, 1985). This protein was found to have two cysteine residues, Cys-199 and Cys-208, which,



in the oxidized form of the protein, formed a disulfide. This leads to a substantial conformational change in the C-terminal domain and activates the transcription of enzymes important in the response to oxidative stress (Choi *et al.*, 2001).

In higher eukaryotes, it has been shown that exposure to electrophiles and ROS leads to a coordinated response regulated through a cis-acting element called the antioxidant response element (ARE) within the regulatory region of target genes (Rushmore *et al.*, 1991). Genes encoding a subset of drug metabolising enzymes, such as GSTs (Goretzki *et al.*, 1990) and NAD(P)H-quinone oxidoreductase 1 (NQO1) (Rushmore *et al.*, 1991), have been shown to be under ARE regulation, along with a subset of anti-oxidant genes such as haem oxygenase-1 (HO-1) (Alam *et al.*, 1995), the subunits of  $\gamma$ -glutamylcysteine synthase ( $\gamma$ -GCS) and thioredoxin (Kim *et al.*, 2001). Nrf2 (NF-E2 related factor 2) was identified as the major regulator of ARE mediated gene expression (Ishii *et al.*, 2002). It is a member of the “cap’n’collar” (CNC) family of transcription factors, which also includes NF-E2, Nrf1, Nrf3, Bach1 and Bach2 (Chan *et al.*, 1993; Oyake *et al.*, 1996). This family of basic leucine zipper transcription factors (bZIP) forms heterodimers with another family of bZIP, small maf proteins, which consist of MafK, MafG and MafF, and then associate with various maf recognition element (MARE) related sequences, including ARE (Itoh *et al.*, 1997). Depending on which CNC forms a heterodimer with a small maf protein transcription is either positively (in the case of Nrf/Maf) or negatively (in the case of Bach/Maf) regulated. Nrf2 is controlled by its binding to a cysteine-rich protein Keap1 (Kelch-like ECH associated protein 1) in the cytoplasm. Cul3 binds directly to the BTB domain (named BTB for *Drosophila* broad-complex C, Tramtrack, and Bric-a-brac) of Keap1 to form a BTB-Cul3-Roc1 E3 ubiquitin ligase which promotes ubiquitination of Nrf2 and subsequent degradation by the proteasome (Fig 1.4;



**Figure 1.4 Activation of ARE**

The KEAP1-CUL3-ROC1 complex promotes Nrf2 ubiquitination and subsequent degradation by the proteasome. ROS or phosphorylation of Nrf2 causes release of Nrf2 and allows it to translocate to the nucleus to form heterodimers with small maf proteins and upregulate genes with an ARE. Bach1 also forms heterodimers with small maf proteins and down regulates transcription by binding to ARE. Heme binds to Bach1 changing its conformation and releasing it.

Furukawa *et al.*, 2005). This redox-sensitive interaction is disrupted on stimulus allowing nuclear accumulation of the Nrf2 protein (Itoh *et al.*, 2003). Keap1 in the mouse has 25 cysteine residues, of which, four are sensitive to alkylation. These cysteines (Cys-257, Cys-273, Cys-288 and Cys-297) are located in the intervening space between the two protein-binding domains in Keap1 and their modification could lead to substantial conformational changes, resulting in dissociation from Nrf2 (Dinkova-Kostova *et al.*, 2002). It has been shown that electrophilic lipids can activate ARE through thiol modification of Keap1 using 15-deoxy-prostaglandin J<sub>2</sub> (Levonen *et al.*, 2004). These electrophilic lipids are substrates for GSTs, in particular GST Pi. It has also been suggested that phosphorylation of Nrf2 on serine-40 by protein kinase C can also cause its release (Huang *et al.*, 2002) and translocation to the nucleus. Whatever the process, it is the release of Nrf2 from Keap1 that is the important step in the nuclear build up of Nrf2 as shown using siRNA against Keap1, which down-regulated Keap1 and caused nuclear translocation of Nrf2 (Devling *et al.*, 2005). Keratinocyte growth factor (KGF) expression is strongly increased in wounded or inflamed tissue and is important for the repair process. Nrf2 has been shown to be controlled on a transcriptional level by KGF, increasing its expression (Braun *et al.*, 2002) and this may be at least partially responsible for the cytoprotective effect of KGF.

H<sub>2</sub>O<sub>2</sub> is one of the ROS generated as a by-product of cellular metabolism and as a result of exposure to UV and other environmental agents. ROS also work as second messengers in cells and are required for optimal activation of numerous signalling pathways including those mediated by protein tyrosine kinases. They do this through transient inactivation of protein tyrosine phosphatases (PTPs) (Denu *et*



*al.*, 1998). Compared to phosphorylation in general, phosphorylation on tyrosine is extensively used only in multicellular eukaryotes. Tyrosine phosphorylation is used for communication between and within cells, to determine the shape and motility of cells, in decisions to proliferate or differentiate, and in cellular processes like gene transcription and RNA processing (Mustelin *et al.*, 2002). This process is controlled by altering the balance between protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTPs). The importance of PTPs is seen in their roles in many human diseases including allergy, asthma, obesity, multiple sclerosis, myocardial hypertrophy and Alzheimer's disease (Bottini *et al.*, 2002; Anderson *et al.*, 2004). Type 2 diabetes and obesity are multifactorial diseases in which insulin resistance is a key feature. PTPs are involved in negative regulation of insulin signaling, and PTP1B has been implicated in diabetes and obesity. Studies generating PTP1B knockout mice have demonstrated that removal of PTP1B not only increases insulin sensitivity but also produces resistance to diet induced obesity (Elchebly *et al.*, 1999). CD45 is an abundant transmembrane receptor-like PTP that is expressed exclusively on haemopoietic cells and plays a positive role in promoting signaling through T and B cells (Penninger *et al.*, 2001). A polymorphism in CD45 has been reported to be associated with multiple sclerosis (Jacobsen *et al.*, 2000). PTPs are cysteine-dependent enzymes defined by a PTP signature motif, Cys-(X<sub>aa</sub>)<sub>5</sub>-Arg, at their catalytic site (Barford *et al.*, 1995). It is the cysteine that is essential for activity, forming a thiol-phosphate intermediate in the dephosphorylation reaction (Fauman *et al.*, 1996). As the cysteine residue has a low pK<sub>a</sub>, making it susceptible to oxidation and inactivation of the enzyme, therefore PTPs may serve as sensors of cellular redox state. A member of this family of phosphatases is the low molecular weight protein tyrosine phosphatase (LMW-PTP), which has been shown to function in the down

regulation of platelet-derived growth factor and insulin receptors (Chiarugi *et al.*, 1995). It is the sole PTP to have two cysteines in the active site motif; Cys-12, which is the catalytic cysteine and Cys-17. Both of these cysteines are conserved in all prokaryotic and eukaryotic organisms, indicating their importance. After treatment with  $\text{H}_2\text{O}_2$ , Cys-12 and Cys-17 are found to form a disulfide bond and inactivate the enzyme (Caselli *et al.*, 1998). In the case of protein tyrosine phosphatase B1 (PTPB1), it was shown that the inactivation of the enzyme was caused by the formation of a cyclic sulfenyl-amide after oxidation by  $\text{H}_2\text{O}_2$ , in which the sulfur of the catalytic cysteine is covalently linked to the main chain nitrogen of the adjacent serine residue (Salmeen *et al.*, 2003). Another member of the PTP family, receptor protein-tyrosine phosphatases (RPTP), forms an intermolecular disulfide bond in RPTP dimers, leaving them in an inactive conformation after treatment with  $\text{H}_2\text{O}_2$  (van der Wijk *et al.*, 2004). In the formation of the sulfenyl-amide or disulfide bond there is the elimination of oxygen from sulfenic acid and the suppression of further reversible oxidation. These modifications may be reversed by rapid reaction with glutathione, forming *S*-thiolate derivatives, followed by reductive processes that include direct reduction by glutathione, or more likely, by dithiol redox proteins such as thioredoxin or glutaredoxin.

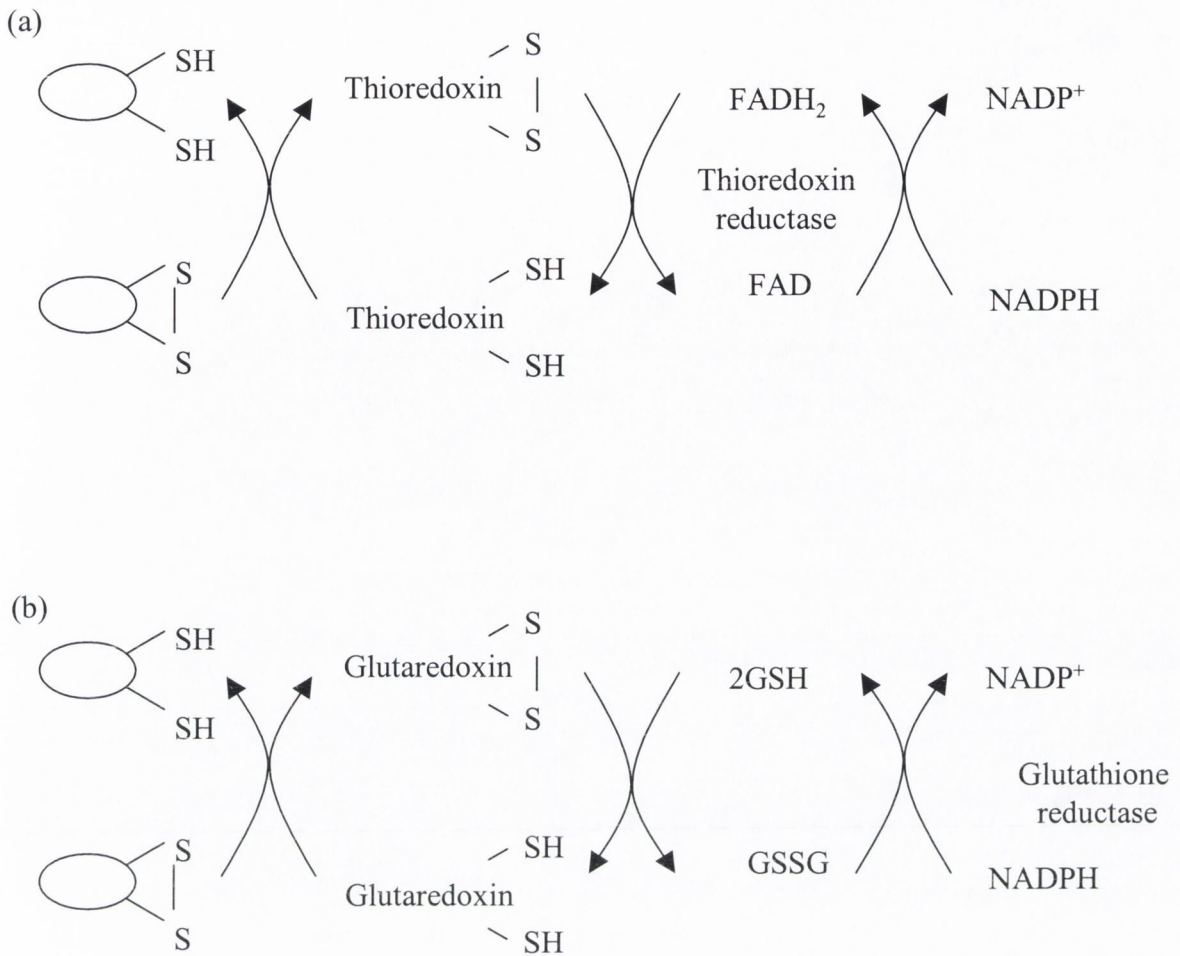
Under conditions of oxidative stress, glutathionylation (the covalent attachment of glutathione to protein cysteines through a mixed disulfide bond) can occur (Klatt *et al.*, 2000). There are several ways in which a protein may be glutathionylated, e.g. oxidized glutathione (GSSG) can react with a protein thiol or a previously oxidized cysteine sulfenic acid can react with reduced glutathione (GSH). In many cases, oxidative-stress-induced glutathionylation precedes any major change in the GSH/GSSG ratio, so the second pathway may be the more relevant (Thomas *et*



*al.*, 1995). Nitrosation reactions with *S*-nitrosoglutathione (GSNO) may also be accompanied by protein thiol *S*-thiolation (Mohr *et al.*, 1999). Glutathionylation of protein thiols has been implicated in many processes, the first being protection. Glutathionylation of thiols that have been oxidized to sulfenic acid prevents further irreversible oxidation and, when stress levels decrease, the glutathione can be removed by thioredoxin, glutaredoxin or small thiols such as GSH. In addition some exposed, non-catalytic protein thiols may help buffer the GSH/GSSG ratio by reacting with GSSG to release one or two GSH molecules, leaving a protein mixed disulfide or a protein disulfide. This would maintain the glutathione pool during transient oxidative stress.

More recently there has been a lot of evidence for glutathionylation in regulation under conditions of oxidative stress. It has been shown that treatment of the yeast *Saccharomyces cerevisiae* with H<sub>2</sub>O<sub>2</sub> causes *S*-glutathionylation-induced inactivation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase and alcohol dehydrogenase. This inactivation is reversible once the oxidant has been removed. Hydrogen-peroxide induced stress has no effect on glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase (Shenton *et al.*, 2003), enzymes that catalyse NADPH production via the pentose phosphate pathway. This would lead to glucose equivalents entering the pentose phosphate pathway for the generation of NADPH, which, in turn, supports reduction of GSSG by the NADPH-dependent glutathione reductase. During exposure to oxidants, NADPH is important since it provides the reducing power for anti oxidant enzymes such as the thioredoxin system (Fig. 1.5; Holmgren *et al.*, 1989). *S*-Glutathionylation of GAPDH is also observed during heart reperfusion experiments (Eaton *et al.*, 2002).





**Figure 1.5 Reactions of the thiol-disulfide oxidoreductase family**

These enzymes are involved in the formation and elimination of disulfide bonds through the catalysis of thiol disulfide interchange reactions.

(a) The thioredoxin system consists of thioredoxin reductase and thioredoxin.

(b) The glutaredoxin system is composed of glutathione reductase, glutathione and glutaredoxin.

The transfer of extracellular signals through the cytoplasm and into the cell nucleus is driven by phosphorylation cascades, involving the mitogen-activated protein kinases (MAPK), MAPK kinases (MAPKK) and MAPKK kinases (MAPKKK) as well as various Ser/Thr protein kinases, such as cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). Oxidative stress and glutathionylation also play a role in the MAP kinase cascades. Various physiological events stimulate the production of ROS and these stress signals activate JNK, a stress activated protein kinase (SAPK) (Hibi *et al.*, 1993). JNK is activated upstream by both MAPK/extracellular signal-regulated kinase kinase (MEKK1) and ASK1, through their phosphorylation of MAP kinase kinase 4 (MKK4), which acts on JNK (Yan *et al.*, 1994; Ichijo *et al.*, 1997). MEKK1 appears to transmit a survival signal (Cardone *et al.*, 1997) whereas ASK1 has a clear role in signalling JNK during stress induced cell death (Takeda *et al.*, 2003). These similar protein kinases with apparently identical activities in phosphorylation of downstream target substrates have opposing functions in the cell. However they are not controlled just by phosphorylation. Under conditions of oxidative stress, MEKK1 is inhibited by site-specific glutathionylation at the ATP binding domain at Cys-1238 (Cross *et al.*, 2004). ASK1 is inhibited by thioredoxin (Trx) by direct binding with the N terminal region of ASK1 (Saitoh *et al.*, 1998) and ROS such as  $H_2O_2$  which cause glutathionylation of Trx, causes its dissociation from ASK1 and thereby activates ASK1 by inducing oligomerization and subsequent phosphorylation of a critical threonine residue within the activation loop of ASK1 (Tobiume *et al.*, 2002). Protein serine/threonine phosphatase (PP5) binds specifically to the activated form of ASK1 in response to oxidative stress and causes dephosphorylation, and therefore inactivation, of ASK1 as a negative-feedback mechanism (Morita *et al.*, 2001). Transient and sustained activations of JNK have

been implicated in cell survival and apoptosis, respectively, so this may play a critical role in making decisions about the fate of cells via the MAP kinase pathways, in which the redox state of the cell and glutathionylation could be important factors.

Another signalling pathway influenced by glutathionylation is the cAMP-dependent protein kinase (PKA) pathway. PKA is a ubiquitously expressed signalling molecule that is essential in numerous metabolic processes and has been implicated in a wide range of cellular activities, including glycogen metabolism, cell proliferation, differentiation and apoptosis (Shabb *et al.*, 2001). In the absence of cAMP, the kinase exists as an inactive tetramer comprising two catalytic subunits (C) and two regulatory subunits (R). Compared to many protein kinases that are activated by transient phosphorylation, PKA appears to be assembled in an active, phosphorylated state and kept inactive by the formation of a complex with the R-subunits (Yonemoto *et al.*, 1993). When cAMP binds to the R-subunits, it releases the active C-subunit, which, in turn, can be inactivated by the binding of PKI (Wen *et al.*, 1994). PKA is phosphorylated at Thr-197. Removal of this phosphate was not thought of as a regulatory mechanism as early studies showed that Thr-197 was constitutively phosphorylated and that it was also very resistant to phosphatase treatment (Yonemoto *et al.*, 1993). However, it has been shown recently that glutathionylation of a reactive cysteine, Cys-199 on the activation loop of PKA, enhances the ability of the kinase to be dephosphorylated at Thr-197, thereby inactivating the kinase activity. It has also been shown that the kinase is preferentially rephosphorylated by 3-Phosphoinositide Dependent Protein Kinase-1 (PDK1) when the cysteine is reduced (Humphries *et al.*, 2004). PKA is a member of the AGC family of kinases, each of which contains a highly conserved cysteine in their activation loop. Thiol



|                                          |                                    |
|------------------------------------------|------------------------------------|
| 1-CYS peroxiredoxin                      | Manevich <i>et al.</i> , 2004      |
| $\alpha$ -crystallin                     | Craghill <i>et al.</i> , 2004      |
| Actin                                    | Dalle-Donne <i>et al.</i> , 2003   |
| Aldolase                                 | Offermann <i>et al.</i> , 1984     |
| cAMP dependent protein kinase A          | Humphries <i>et al.</i> , 2002     |
| Annexin A2                               | Caplan <i>et al.</i> , 2004        |
| Carbonic anhydrase III                   | Mallis <i>et al.</i> , 2000        |
| c-jun                                    | Klatt <i>et al.</i> , 1999         |
| Creatine kinase                          | Reddy <i>et al.</i> , 2000         |
| Glyceraldehyde-3-phosphate dehydrogenase | Mohr <i>et al.</i> , 1999          |
| H-ras                                    | Mallis <i>et al.</i> , 2001        |
| HSP27                                    | Eaton <i>et al.</i> , 2002b        |
| MEKK1                                    | Cross <i>et al.</i> , 2004         |
| Mitochondrial complex 1                  | Taylor <i>et al.</i> , 2003        |
| NF- $\kappa$ B                           | Pineda-Molina <i>et al.</i> , 2001 |
| Protein kinase C $\alpha$                | Ward <i>et al.</i> , 2000          |
| Protein kinase D                         | Chu <i>et al.</i> , 2001           |
| Protein phosphatase 2A                   | Rao <i>et al.</i> , 2002           |
| Protein tyrosine phosphatase 1B          | Barrett <i>et al.</i> , 1999       |
| Thioredoxin                              | Casagrande <i>et al.</i> , 2002    |
| Tubulin                                  | Landino <i>et al.</i> , 2004       |

**Table 1.1    Examples of proteins that are known to be glutathionylated.**

modification may be a general mechanism for enhancing dephosphorylation in other members of this family, as has been shown to be the case with PKC $\alpha$  which has also shown to be glutathionylated (Ward *et al.*, 2000).

In the mitochondria, superoxide is generated and converted to H<sub>2</sub>O<sub>2</sub>, which can diffuse from the mitochondria into the cytoplasm (Finkel *et al.*, 2000). The redox active thiols of the 51 and 75 kDa subunits of complex 1 react with GSSG to form a mixed disulfide with glutathione. These subunits are the entry site for electrons from NADH into complex 1 (Hirst *et al.*, 2003). In addition, the selective cleavage of the 75 kDa subunit by caspases is an important early event in apoptosis (Ricci *et al.*, 2004). Glutathionylation of complex 1 was found to lead to a rapid increase in superoxide formation, which could be reversed by removal of the glutathione (Taylor *et al.*, 2003). The formation of mixed disulfides with glutathione in complex 1 may have physiological significance. Complex 1 is much more susceptible to glutathionylation than the other complexes in the respiratory chain and it is selectively damaged in Parkinson's disease and Huntington's disease (Cooper *et al.*, 1992). This mechanism of reversible mitochondrial ROS production suggests a mechanism by which mitochondria might also regulate redox signalling.

A different form of control is seen in carbonic anhydrase isoenzyme III (CAIII). The carbonic anhydrases catalyse the reaction of carbon dioxide with water to form carbonic acid (Dodgson *et al.*, 1991). CAIII is distinguished from the other isoenzymes by several characteristics, particularly by a lower specific activity (Eriksson *et al.*, 1993). CAIII is found in large amounts in certain tissues, approximately making up 5% of soluble protein in rat liver, 8% in skeletal muscle and up to 25% in adipocytes. It also has a CX<sub>5</sub>R phosphatase motif, C<sup>181</sup>LFPAC<sup>186</sup>R. Cys-181 is at the centre of a distinctive phosphate-binding loop, facilitating formation of

the phosphocysteine intermediate that is essential to the catalytic cycle of a phosphatase. Glutathionylation of Cys-186 is required for phosphatase activity, but a second glutathionylation at Cys-181 inhibits the activity (Cabiscol *et al.*, 1996). So, this enzyme's phosphatase activity requires the first glutathionylation but is inactivated by either a second glutathionylation or by the removal of the glutathione.

Depletion of GSH in the lens, with a concomitant increase in protein thiol mixed disulfide, is associated with the normal aging process (Lou *et al.*, 1990). The crystallins, which are the structural proteins of the lens, make up 90% of the total protein.  $\alpha$ -Crystallin plays an important role as a chaperone and its glutathionylation decreases this activity which can, in turn, affect the stability of other crystallins and the state of transparency of the whole lens, causing lens opacification (Cherian *et al.*, 1997).

### 1.3 GSTP1-1

Interest in GSTP1-1 is in large part connected to cancer, as it was shown to be a marker for preneoplastic foci in rats (Sato *et al.*, 1985). Immunochemical and stereological analyses revealed that the level of enzyme in preneoplastic foci in rat hepatocytes was 150-250-fold higher than that found in normal cells (Sato *et al.*, 2002). GSTP1-1 is expressed in most human tissues, especially in placenta and red cells but not in adult liver (Guthenberg *et al.*, 1981). Increased expression levels of GSTP1-1 are associated with malignant transformation and tumour development in a wide range of tissues (Monden *et al.*, 1997). The detoxifying activity of GSTP1-1 is in part responsible for the development of cellular multi-drug resistance towards a number of chemotherapeutic agents (O'Brien *et al.*, 1996). The over-expression of GSTP1-1 in tumour cells promotes the conjugation of GSH to alkylating cytostatic



drugs, leading to their inactivation and extrusion from the cell by means of specific efflux pumps (Keppler *et al.*, 1999). To counteract this phenomenon, specific inhibitors of GSTP1-1 have been designed. Ethacrynic acid, for example, has been found to sensitise tumour cells to the cytotoxic effect of alkylating agents and, in vivo, to enhance the effect of chlorambucil in patients with chronic lymphoblastic leukaemia (Tew *et al.*, 1997). There are side effects that limit its clinical utility and more specific inhibitors to GSTP1-1 such as the glutathione derivative TER199, are being developed to help sensitize tumours over expressing the enzyme.

It has also been shown that over-expression of GSTP1-1 in neurons protects them from dopamine induced apoptosis (Ishisaki *et al.*, 2001). It has been suggested that dopamine induces apoptosis through an oxidant dependent JNK activation pathway. A dominant negative mutant, SEK-1, a kinase that phosphorylates JNK, prevented dopamine-induced activation and apoptosis in primary neonatal, postmitotic and striatal neurons (Luo *et al.*, 1998). GSTP1-1 may in part mediate its effect through its anti-oxidative activity but it has also been identified as a JNK inhibitor (Adler *et al.*, 1999).

A lack of GSTP1-1 can also have a significant impact on cells, as shown in GSTP1-1 null mice, which were shown to be more susceptible to carcinogens. Mouse embryo fibroblasts from these null mice doubled their population in 26.2 h versus 33.6 h for the wild type. Treatment with a GSTP1-1 specific inhibitor, TLK199 stimulated both lymphocyte production and bone marrow progenitor proliferation, but only in the wild type mice. This suggests a role for GSTP1-1 in cell proliferation (Ruscoe *et al.*, 2001). Hypermethylation of CpG island sequences in the promoter region of GSTP1-1 is responsible for the transcriptional silencing of this protein and is seen in a number of cancers. For example, it is the most common somatic genome

alteration described for human prostate cancer (Lee *et al.*, 1994) and lack of GSTP1 expression is characteristic of human prostate cancer cells in vivo (Lin *et al.*, 2001). This is also seen in some forms of hepatocellular carcinomas (Zhang *et al.*, 2005). The hypermethylation of GSTP1 promoter has been suggested as a potential diagnostic marker for prostate cancer (Bernardini *et al.*, 2004).

GSTP1-1 has been shown to have an important role in the binding of non-substrate ligands. Carotenoids are ubiquitous coloured pigments synthesized by plants and microorganisms, but not by higher organisms. Higher organisms ingest carotenoids in their diet and subsequently utilize them for important physiological functions. Zeaxanthin (a xanthophyll carotenoid that screens against blue light) is found in the retina, and high macular concentrations of this antioxidant are associated with decreased risk of age-related macular degeneration (Bernstein *et al.*, 2002), the leading cause of blindness in the developed world. The uptake, metabolism and stabilization of xanthophyll carotenoids in the retina are thought to be mediated by specific xanthophyll binding proteins (XBPs). A XBP was purified from human macula and shown to be GSTP1-1 (Bhosale *et al.*, 2004). Binding of 15-d-PGJ<sub>2</sub>, a terminal metabolite of the J-series cyclopentone prostaglandins, by GSTP1-1 has been shown to inhibit its effect as a ligand for the peroxisomal proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ; Paumi *et al.*, 2004). Activation of PPAR $\gamma$  by binding this ligand facilitates PPAR $\gamma$ /retinoid X receptor heterodimer formation, recruitment of transcriptional coeffectors and binding to its PPAR responsive elements. This ligand binding is associated with the altered expression of genes including those involved in adipocyte differentiation, cell proliferation, and lipid and glucose homeostasis (Chawla *et al.*, 2001).



#### 1.4 GST Pi reactive thiols

Human, pig and rat GSTP1-1 contain four cysteines per monomer at positions 14, 47 (45 in the pig), 101 and 169 (Cowell *et al.*, 1998; Okuda *et al.*, 1987; Dirr *et al.*, 1991). Mouse GSTP1-1 has only three cysteines at 14, 47 and 169 making it a simpler system for studying thiols. GSTP1-1 was shown to be almost completely inactivated by treatment with *N*-ethylmaleimide (NEM) which reacts with thiol groups, but has little or no effect on the activity of GSTs from the Alpha or Mu classes under the same conditions. Inactivated GSTP1-1 lost its ability to bind to *S*-hexylglutathione-Sepharose. The site of inactivation of rGSTP1-1 by NEM was identified as Cys-47 (Tamai *et al.*, 1990). Lo Bello *et al.* (1990) also identified the site of inactivation of hGSTP1-1, using NEM, as Cys-47.

CDNB reacts irreversibly with Cys-47 in hGSTP1-1 and inactivates activity. The protein is protected when GSH or *S*-methylglutathione is included in the reaction (Caccuri *et al.*, 1992). Desideri *et al.* (1991) showed, using a spin-labelled alkylating reagent and electron paramagnetic resonance spectroscopy (EPR), that there is a reactive thiol group near the catalytic site of hGSTP1-1. Their result indicates the possible occurrence of a structural rearrangement after binding of the maleimide spin label. Human GSTP1-1 was found to be inactivated by the biological disulfides cystine and cystamine with pseudo first order kinetics, but oxidized glutathione, GSSG, did not react with Cys-47 (Nishihara *et al.*, 1991).

Philips *et al.* (1993) showed mGSTP1-1 modified at Cys-47 by ethacrynic acid or DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] is inactive, but that replacement of NTB (nitrothiobenzoate) on Cys-47 by the less bulky cyanide residue partially restored activity. This suggests that the bulk of the modifying reagent is important. A



similar result was found for the modification of rGSTP1-1 using methylmethane thiosulfonate (Nishihira *et al.*, 1992). Cys-101 was also modified by this reagent but that modification had no effect on the activity of the enzyme.

Rat and human (Shen *et al.*, 1991) and pig (Sluis-Cremer *et al.*, 1995)

GSTP1-1 were found to be inhibited by hydrogen peroxide. The inactive proteins could be reactivated in the presence of a partially purified thioltransferase (glutaredoxin) but not a thioredoxin (Terada *et al.*, 1993). The peroxide treated enzyme showed extra bands on SDS-PAGE under non reducing conditions. It was suggested that these extra bands were caused by intrasubunit disulfide bonds (Shen *et al.*, 1993).

When the Cys-47 in rGSTP1-1 was changed to an alanine the mutant enzyme became resistant to inactivation by NEM and physiological disulfides, such as cystamine or cystine (Tamai *et al.*, 1991). When all cysteines were changed to alanine individually in rGSTP1-1, there was no change in the specific activity compared to that of the wild type protein. Kong *et al.* (1991) reported that the specific activity dropped by 40% in a C47S mutant of hGSTP1-1 and the apparent  $K_M^{GSH}$  increased from 150  $\mu$ M for the native enzyme to 760  $\mu$ M for the C47S mutant. Lo Bello *et al.* (1993) suggested that the reactivity of Cys-47 is caused by the thiol forming an ion pair with Lys-54. They determined that the pKa of the Cys-47 is 4.2 using pH dependence of a UV absorption peak at 229 nm, which was attributed to a thiolate anion. The same group found that the C47A and C47S mutants of hGSTP1-1 show positive cooperativity with respect to GSH. Hill coefficients of 1.3 and 1.4 were measured for the two mutants, C47A and C47S, respectively. The K54A hGSTP1-1 mutant was also found to exhibit positive cooperativity towards glutathione and the pKa for the thiolate ion of Cys-47 increased to 5.4. No cooperative effect was found

for the hydrophobic substrate CDNB (Lo Bello *et al.*, 1995; Ricci *et al.*, 1995). The pKa for the Cys-47 in mGSTP1-1 was determined to be 7.2 by measuring ethacrynic acid inactivation at different pH values (Phillips *et al.*, 1993).

Mutation of the Cys-14 residue to a serine in hGSTP1-1 indicated that Cys-14 contributes to the catalytic activity and also to some extent to the binding of glutathione, with the apparent  $K_M$  increasing 1.7-fold (Park *et al.*, 2001). Based on the three-dimensional structure of hGSTP1-1, no interaction is expected between Cys-14 and the enzyme-bound GSH (Reinemer *et al.*, 1992). Furthermore, Tyr-7 is located in a hydrophobic pocket formed by the side chains of Phe-8, Val-10, Gly-12, Cys-14 and Pro-53, and is only partially exposed to solvent (Dirr *et al.*, 1994). An analysis of the electrostatic environment at the active site of GSTP1-1 also indicates that the peptide dipoles at Gly-12, Arg-13 and Cys-14 generate an extremely high positive electrostatic potential at the position of the hydroxyl group of Tyr-7 (Karshikoff *et al.*, 1993).

To summarize, although no cysteine residues take part directly in the enzyme's active site, they do appear to play a role in the enzyme's activity. It is proposed that Cys-47 may act as a sensor for oxidative stress, whereby in the absence of glutathione, Cys-47 is more susceptible to oxidation and inactivation of the enzyme occurs. The importance of the other two cysteines in mGSTP1-1, Cys-14 and Cys-169, is not well understood and further study is required.

## 1.5 Aims of this study

- (1) To generate all of the mutants of mGSTP1-1 with cysteine to alanine substitutions so we can study in greater detail the importance of the thiols in function of the enzyme, which has been shown to be important in the treatment of many cancers.
- (2) To use DTNB on the various cysteine to alanine mutants to study the three different cysteines. Cys-47 which has already been described as a reactive thiol with a pKa around 7, that is found on the surface of the protein and is near the active site. Cys-169 that has a high pKa and although it is on the surface is not very reactive so is not seen as very important. And Cys-14 which is buried under the active site and its reactivity is not very well understood.
- (3) To compare the initial rate kinetics of the different cysteine to alanine mutants, as previous work has presented limited data, referring simply to apparent  $K_m$  and apparent  $V_{max}$  values.
- (4) To study aspects of glutathionylation reactions in detail using mono-thiolated and non-thiolated mutants of mGSTP1-1. To facilitate this study a new reagent, glutathionyl-*S-S*-nitrobenzoate (GSSN), the mixed disulfide between GSH and NTB, needs to be synthesised and characterised.



# Chapter 2

## 2.1 Materials

Biochemical reagents and chemicals used were of analytical grade, where possible, and obtained from BDH, Reidel-de Haen and Sigma. The names and addresses of suppliers can be found in the appendix.

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| Materials                         | Source               |
|-----------------------------------|----------------------|
| Acrylamide/Bis Solution           | National Diagnostics |
| Agarose (molecular biology grade) | Promega              |
| Agarose Gel DNA Extraction Kit    | Boehringer Mannheim  |
| Ampicillin, sodium salt           | Sigma                |
| Bacto-Tryptone                    | Difco                |
| Bacto-Yeast                       | Difco                |
| Bacto-Agar                        | Difco                |
| 1-Chloro-2,4-dinitrobenzene       | Sigma                |
| 4-Chloronaphthol                  | Sigma                |
| Dimethylsulfoxide                 | Sigma                |
| DNA molecular weight marker VII   | Boehringer Mannheim  |
| dNTPs (100 $\mu$ M stock)         | Sigma                |
| Ecoscint                          | National Diagnostics |
| Folin and Ciocalteau's reagent    | Sigma                |
| Freund's adjuvant, complete       | Sigma                |

|                                               |               |
|-----------------------------------------------|---------------|
| Freund's adjuvant, incomplete                 | Sigma         |
| Glutathione, reduced                          | Sigma         |
| Lysozyme                                      | Sigma         |
| Nitrocellulose paper                          | Sigma         |
| Oligonucleotide primers                       | MWG Biotech   |
| Prestained Molecular Markers SDS-7B           | Sigma         |
| Restriction enzymes                           | Roche/Promega |
| <i>S</i> -( <i>p</i> -nitrobenzyl)glutathione | Sigma         |
| Sephadex G-25                                 | Sigma         |
| Sepharose 6B                                  | Sigma         |
| T4 DNA Ligase                                 | Promega       |
| Taq DNA polymerase                            | Promega       |



## **2.2 Purification of mouse Glutathione *S*-Transferase P1 and mutants**

### **2.2.1 Preparation of GSH affinity resin**

#### **2.2.1.a Epoxy-activation of Sepharose**

Sepharose 6B (250 ml) was washed with water (2 l) on a sintered glass funnel. The resin was mixed with 0.6 M NaOH (250ml) containing NaBH<sub>4</sub> (0.75 g) and 1,4-butanedioldiglycidyl ether (250 ml), in a fume hood. The reaction was left stirring gently in a fume hood overnight at room temperature. The resin was rinsed extensively with water (6 l) on a sintered glass funnel.

#### **2.2.1.b Coupling of glutathione to epoxy-activated-sepharose**

Glutathione-Sepharose was synthesized by the method of Simons & Vander Jagt (1977). Epoxy-activated-Sepharose 6B (250 ml) was washed with 2 l of water on a sintered glass funnel. The gel was washed with 1 l of sodium phosphate, pH 7, prepared by mixing 80 ml of 0.2 M NaH<sub>2</sub>PO<sub>4</sub> and 280 ml of 0.1 M Na<sub>2</sub>HPO<sub>4</sub> with 640 ml of water. The resin was transferred to a conical flask and the volume was adjusted to 400 ml with the same buffer. Nitrogen was passed through the suspension for 5 min. Glutathione (8 g) dissolved in 80 ml of water (with the pH adjusted to 7 using 5 M KOH) was added and the mixture was stirred gently at 37°C for 24 h.

The gel was washed with 2 l of water and the remaining active groups were blocked by allowing the gel to stand in 1 M ethanolamine for 4 h at room temperature.

The glutathione-Sepharose was washed sequentially with 1 l each of 0.5 M KCl in 0.1 M sodium acetate (pH 4), 0.5 M KCl in 0.1 M sodium borate (pH 8) and finally with 20 mM sodium phosphate (pH 7).

### **2.2.2 Expression of recombinant mGSTP1 in *E. coli*.**

For details on the construction of the various expression plasmids used, see Sections 2.6, 2.7 and 2.8. When purifying the recombinant product frozen *E. coli* stocks (transformed with the appropriate pET11A vector) were streaked out on agar plates (containing 100 µg/ml ampicillin) and incubated overnight at 37°C. A single colony was streaked out and incubated on a new agar plate to produce an adequate number of single colonies. Single colonies from these plates were used to inoculate LB broth. (containing 100 µg/ml ampicillin). The bacteria were grown overnight at 37°C with shaking at 270 rpm. The overnight culture was transferred to 2 x 500 ml LB broth containing 100 µg/ml ampicillin. The cells were grown at 37°C with shaking at 270 rpm and the growth rate was monitored by measuring the absorbance at 600 nm. When an optical density (OD) of between 0.4 and 0.6 had been reached, the expression of mGSTP1 wild type and of the various mutants was induced by adding IPTG (0.2 mM final concentration). The bacteria were incubated at 37°C with shaking at 270 rpm for a further 4 h. The cells were harvested by centrifugation at 7700 *g* for 15 min at 4°C in a GSA rotor. The supernatant was decanted and the pellets were frozen overnight.

The pellets were resuspended in 30 ml of PBS buffer (10m M sodium phosphate, 0.9% sodium chloride, pH 7) and transferred to a 50 ml centrifuge tube. A 1ml volume of lysozyme (10 mg/ml) was added to break open the bacterial cell walls. The bacterial suspension was left on ice for 30 min and then sonicated using a

soniprobe type 7532A from Dawe Instruments. The sonicator was initially washed with ethanol and then with distilled water. The centrifuge tube was placed in a beaker of ice to prevent heating during the process. The probe was lowered into the suspension and the sonicator was turned to a setting of 100 Watts. Sonication was then carried out for 5 s bursts with 5 s intervals for 5 min. The probe was once again washed with ethanol and distilled water. The sonicated suspension was then centrifuged at 19,000 g for 45 min at 4°C using the SS34 rotor. The supernatant was decanted into a 50 ml falcon tube and kept on ice.

### **2.2.3 Affinity Chromatography using Glutathione-Sepharose**

Bacterial lysate was loaded onto a glutathione-Sepharose column (normally 5 ml resin per litre of bacterial culture) at 4°C, pre-equilibrated with PBS [10 mM sodium phosphate, 0.9% (w/v) NaCl, pH 7]. When the lysate was loaded, the column was washed with PBS until the absorbance at 280 nm was less than 0.01. The material bound to the column was then eluted with 5 mM GSH in 10 mM Tris-HCl, pH 8. The elution of protein was followed by measuring the absorbance at 280 nm.

### **2.2.4 Protein determination**

For routine column monitoring, the absorbance at 280 nm was measured and a value of 1 was assumed to be equivalent to a protein concentration of 1 mg/ml.

For more accurate protein determination the method of Markwell *et al.* (1978) was used. Bovine serum albumin (BSA) was used as a protein standard. Reagent C was made by mixing 100 ml of reagent A [2% (w/v) sodium carbonate, 0.4% (w/v)



sodium hydroxide, 0.16% (w/v) potassium tartrate, 1% (w/v) SDS] and 1 ml of reagent B [4%(w/v) copper sulfate]. The BSA (0-100  $\mu$ g) or unknown protein, in a volume of 0.5 ml was mixed with reagent C (1.5 ml). After incubation 10 min, 50% (v/v) Folin-Ciocalteu solution (0.15 ml) was added and the solutions were mixed. The solutions were left to develop for 45 min and the absorbance was determined at 660 nm using a Molecular Devices Spectra max 340PC plate reader. The absorbances of the standard were fitted to a straight line using a least-squares procedure and this was used to estimate the protein concentration of the unknown samples.

## **2.2.5 Desalting and concentrating protein solutions**

### **2.2.5a Sephadex G-25 gel filtration**

Gel filtration on Sephadex G-25 was used to desalt protein solutions and to separate protein from low-molecular-mass substances. The Sephadex G-25 resin was swollen in water according to the manufacturer's instructions. The column was equilibrated with 10 mM sodium phosphate buffer pH, 7.2, at 4°C and washed with at least 3 column volumes of the same buffer before use. The included and excluded volume of the column were determined using a mixture of 5 mg/ml blue dextran and 10 mg/ml *p*-nitrophenol in sodium phosphate pH 7.2.

The protein samples (maximum of 5% of column volume) were gel filtered using Sephadex G-25 (60 X 2 cm), at a flow rate of no more than 1 ml/min. Fractions (2 ml) were monitored for protein content by measuring the absorbance at 280 nm.

### **2.2.5.b Amicon ultrafiltration**

If required, protein samples were concentrated by centrifugation using a centricon 10. The sample was first centrifuged at 36,000 g for 10 min in an SS34 rotor at 4°C to remove insoluble material and the supernatant was loaded in the centricon, which was centrifuged at 4000 rpm in an SS34 rotor using a Sorval 5C plus centrifuge.

## **2.3 Protein electrophoresis and immunoblotting**

### **2.3.1 SDS-polyacrylamide gel electrophoresis**

SDS-PAGE of GST subunits was carried out using the discontinuous system of Laemmli (1970). Briefly, the procedure used was as follows: the components (Table 2.1) of the resolving gel were poured into the slab gel mould (ATTO) and overlaid with water. When the gel was set, the water was removed, the space between the plates was dried and the stacking gel was poured on top of the resolving gel. A Teflon comb was inserted in the stacking gel which was left to set. Before use the comb was removed and the wells were rinsed with water.

The gel was run in an electrophoresis chamber (ATTO AE-6400 mini-gel system) with the electrode reservoirs filled with running buffer [0.38 M glycine, 0.05 M Tris-HCl and 0.1% (w/v) SDS]. Samples were prepared as described below and loaded into the wells using a Gilson P20 pipette. The electrophoresis chamber was connected to an ATTO Crosspower 500 power pack and the gel was run at a

Table 2.1      Composition of polymerizing solutions for SDS-PAGE (ATTO AE-6400 mini-gel system)

| Stock solutions                           | Stacking gel<br>4% | Resolving gel<br>15% | Resolving gel<br>20% |
|-------------------------------------------|--------------------|----------------------|----------------------|
| 30% acrylamide,<br>0.8% bisacrylamide mix | 0.5 ml             | 5 ml                 | 6.7 ml               |
| 1M Tris-HCl buffer (pH 6.8)               | 380 µl             | -                    |                      |
| 1.5M Tris-HCl buffer (pH 8.8)             | -                  | 2.5 ml               | 2.5 ml               |
| 10% (w/v) SDS                             | 40 µl              | 100 µl               | 100 µl               |
| Water                                     | 2.1 ml             | 2.3 ml               | 0.6 ml               |
| 10% (w/v) Ammonium persulphate            | 30 µl              | 100 µl               | 100 µl               |
| TEMED                                     | 5 µl               | 5 µl                 | 5 µl                 |



constant current of 25 mA until the tracking dye had run off the end of the gel (approximately 2 h).

After electrophoresis was complete, the gel was removed and stained in 30% (v/v) methanol, 10% (v/v) acetic acid and 1.25% (w/v) Coomassie Brilliant Blue R. Gels were destained in 10% (v/v) methanol, 10% (v/v) acetic acid.

The samples were made up in sample buffer [2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue, 1% 2-mercaptoethanol in 25mM Tris-HCl, pH 6.8]. Samples were heated to 100°C for 3 min before loading. Each lane contained between 1 and 10 µg of protein for a pure protein sample and between 100 and 200 µg for cytosol.

When it was necessary to concentrate the protein an equal volume of ice-cold 25% (w/v) trichloroacetic acid (TCA) was added to each protein sample. The mixture was left on ice for 5 min and then centrifuged in a bench-top minifuge (Beckman Microfuge E) for 30 s. The precipitate was washed twice with ice-cold acetone (0.8 ml) and left to dry at room temperature. The protein was dissolved in sample buffer.

## **2.3.2 Western Blotting**

### **2.3.2.a Wet Blotting**

The procedure was that of Towbin *et al.* (1979). SDS-PAGE was performed as described in Section 2.3.1. When electrophoresis was complete, the gel was equilibrated for 15 min in transfer buffer (25 mM Tris-HCl, 192 mM glycine, pH 8.3)

before the protein was electrophoretically transferred from the unstained gel onto nitrocellulose paper using a BioRAD Transblot Cell. The sponges, filter paper and nitrocellulose were all immersed in transfer buffer before the gel and nitrocellulose paper were sandwiched in a Trans-Blot cassette. Care was taken to ensure that no air bubbles were trapped between the gel and the nitrocellulose. The Transblot cassette was placed in a tank filled with transfer buffer and the electrodes were attached to an ATTO Crosspower 500 power pack. Transfer of proteins was achieved using a constant current of 250 mA for 3 h.

#### **2.3.2.b Semi-Dry Blotting**

Western blots were also carried out using a semi-dry blotting apparatus (Semi-Phor, Hoeffer Scientific Instruments, Model TE70). The procedure followed using this system is similar to that described above except that the transfer buffer was 192 mM glycine, 25 mM Tris-HCl, 1.3 mM SDS, 15% (v/v) methanol, pH 8.3, and the transfer was completed after 1 h at a constant current of 110 mA.

#### **2.3.2.c Staining of Western blots**

Following electrophoretic transfer, the nitrocellulose was removed from the cassette and incubated in blocking buffer {TTBS [20 mM Tris-HCl, 500 mM NaCl, pH 7.5 containing 0.05% (v/v) Tween 20] containing 5% (w/v) commercial dried milk product (Marvel)} for 1 hour at room temperature or overnight at 4°C. This solution blocked free protein binding sites on the nitrocellulose paper.

The nitrocellulose paper was rinsed in TTBS and incubated for 1 hour at room temperature with primary antibody (diluted 1:200 with TTBS containing 1% (w/v) Marvel) on a shaking platform. The nitrocellulose paper was washed twice with TTBS for 10 min followed by a 1 h incubation at room temperature with secondary antibody. The secondary antibody used was goat anti-rabbit IgG conjugated to horseradish peroxidase, which was diluted (1:2000, v/v) with TTBS containing 1% (w/v) Marvel. Following incubation, the nitrocellulose paper was washed as described above. The final wash was with 20m M Tris-HCl pH 7.5. For development of the blot with 4-chloronaphthol, the reagent was prepared by mixing two solutions immediately before use. The first solution contained 100 ml of 20 mM Tris-HCl, 500 mM NaCl, pH 7.5 to which 60 µl of hydrogen peroxide was added prior to use. The second solution contained 60 mg of 4-chloronaphthol dissolved in 20 ml of methanol. Colour development occurred within 10 min and was terminated by rinsing the nitrocellulose paper with water. For development of the blot with DAB (3,3'-diaminobenzidine tetrahydrochloride), a DAB tablet from Sigma was dissolved in 5 ml of water and incubated with the nitrocellulose paper. Colour development occurred within 10 min and was stopped as described above.

### **2.3.3 Preparation of antiserum**

#### **2.3.3.a Immunization procedure**

Prior to immunization a blood sample was allowed to clot (as described below) to give pre-immune serum. The purified protein (1 mg) in 10 mM sodium phosphate pH7 (1 ml) was emulsified with an equal volume of Freund's reagent



(complete for primary immunizations and incomplete for boosters). On day one, 1 ml of sample was injected into New Zealand White rabbits. On days 14 and 28, the rabbits received booster injections. A test bleed was carried out 2 weeks after the second booster injection. Approximately 5 ml of blood were taken from the rabbit and the blood was allowed to clot at room temperature for 1 h in a glass vial. The clot was allowed to retract overnight at 4°C and the serum was decanted and centrifuged at 1,000 g to remove any remaining blood cells. A dot blot assay was used to determine the antibody titre of this serum (see Section 2.3.3.b). If the titre was found to be sufficiently high, the rabbits were exsanguinated one week later. The blood collected upon exsanguination was treated in the same manner as the blood sample from the test bleed. The antiserum was frozen in aliquots of 0.5 ml at -70°C.

#### **2.3.3.b Determination of antibody titre**

Antibody titre was determined by dot blotting. Samples of the protein (2 µl containing serial dilutions from 5 µg to 0.5 ng) used for immunization were applied directly onto a piece of nitrocellulose and allowed to air-dry before being developed as described in Section 2.3.2.c.

### **2.4 Kinetic and ligand binding analysis**

#### **2.4.1 Measurement of GST activity**

The GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was measured according to the method of Habig *et al.* (1974). The reaction is based on the

spectrophotometric determination of thioether product formation at 340 nm. Assays were performed at 30°C in assay buffer (100 mM sodium phosphate pH 6.5) containing 1 mM GSH and 1 mM CDNB (unless otherwise stated), in a total volume of 2 ml. Data were collected using a Philips PU8625 spectrophotometer attached to a Kipp and Zonen flatbed chart recorder and a circulating water bath. The CDNB was added from a stock solution (40 mM in ethanol). When this was pipetted into the phosphate buffer, a white suspension was formed that dissolved easily with careful mixing. When the CDNB concentration was varied, the final concentration of ethanol was kept constant at 2.5 % (v/v). The GSH (40 mM) was dissolved in assay buffer and the pH adjusted to 6.5 with 1 M NaOH. The GSH solution was made fresh each day and kept on ice. The assay was started by the addition of protein. The blank rate (buffer instead of protein) was subtracted from the result obtained for each assay. The extinction coefficient of the product [*S*-(2,4-dinitrophenyl)glutathione] is  $9.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  (Habig *et al.*, 1974).

Assays were also performed using a Molecular Devices Spectra max 340PC, controlled by the SOFTmax computer program. The required dilutions of both substrates were prepared in assay buffer and the reaction was started by the addition of protein. The final volume was 200  $\mu\text{l}$  and the protein was added in a 50  $\mu\text{l}$  aliquot to allow for proper mixing.

Conjugation activity towards ethacrynic acid was measured spectrophotometrically at 270 nm. Assays were performed at 30°C in assay buffer (100 mM sodium phosphate pH 6.5) containing 0.25 mM GSH and 0.2 mM ethacrynic acid in a total volume of 2 ml. The extinction coefficient of the product was  $5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ . The blank rate was subtracted from the results obtained from each assay.

#### **2.4.2. Measurement of the binding of ligands to GST using fluorescence quenching**

The fluorescence of protein in 0.1 M sodium phosphate pH 6.5 in a total volume of 2 ml was measured using a Perkin Elmer fluorescence spectrometer (LC 50B) controlled by the Perkin Elmer F1 data manager program. All of the buffers and substrates were made up in distilled water and filtered through glass fibre filters (0.45 µm pore size) to remove particulate material. The excitation wavelength used was 280 nm (slit width 5 nm) and the emission wavelength was measured over the range 300-600 nm (slit width 5 nm).

To determine the effect of ligand binding on protein fluorescence, increasing amounts of the ligand were added and the change in fluorescence was recorded. The excitation wavelength was 280nm (5nm slit width) and the emission wavelength was 340nm (5nm slit width). After correcting for dilution and inner filter effects, the fluorescence change was plotted against the concentration of ligand.

### **2.5 Chemical modification and structural studies**

#### **2.5.1 Inactivation of mGSTP1 and various mutants by ethacrynic acid**

In order to investigate the effect of ethacrynic acid on enzyme activity, enzyme (1-5 mg) was incubated with a range of ethacrynic acid concentrations (0.1-



3 mM). The incubation was carried out at 30°C in 100 mM sodium phosphate pH 6.5. At various times, aliquots of the reaction mixture were assayed for residual activity towards CDNB. The incubation mix was diluted at least 200-fold into the assay buffer and the remaining activity was measured using CDNB as substrate (see Section 2.4.1).

## **2.5.2 Measurement of thiol groups with 5,5'-dithiobis(2-nitrobenzoic acid), Ellman's reagent**

### **2.5.2.a Measurement of free thiol groups**

5,5'-Dithiobis(2-nitrobenzoic acid), DTNB, also known as Ellman's reagent, was used to measure the free thiol groups in proteins (Ellman, 1959). The stock solution of DTNB (10 mM in 100 mM sodium phosphate, pH 6.5) was kept on ice in the dark. Protein (10  $\mu$ M) was incubated with DTNB (200 $\mu$ M) in 100 mM sodium phosphate buffer, pH 7, in a total volume of 200  $\mu$ l. The release of the NTB (nitrothiobenzoate) was measured spectrophotometrically at 412 nm using a Molecular Devices Spectra max 340PC. The reaction was started by the addition of the protein. The number of free thiol groups was calculated using the molar extinction coefficient for NTB of 14,150 M<sup>-1</sup>cm<sup>-1</sup> (Riddles *et al.*, 1979).

### **2.5.2.b. Determination of the pKa of thiol groups**

Reactions were carried out using a Molecular Devices Spectra Max 340PC for the thiol groups with an alkaline pKa that reacted slowly with DTNB. Incubations of protein (10  $\mu$ M) and DTNB (200  $\mu$ M) were carried out in 100 mM boric acid (pH 8-

9.5), 100 mM sodium phosphate (pH 6-8.5) and 100 mM sodium citrate (pH 4.5-6), in a total volume of 200  $\mu$ l. The release of NTB was monitored at 412 nm as described in Section 2.5.2.a.

For the more reactive thiol groups such as cysteine-47, the release of NTB was measured at 412 nm using an Applied Photo Physics Pi star 180 stopped-flow spectrophotometer. DTNB (200  $\mu$ M) in 200 mM boric acid (pH 8-9.5), 200 mM sodium phosphate (pH 6-8.5) and 200 mM sodium citrate (pH 4.5-6) was placed in one syringe and protein (10  $\mu$ M) in 10 mM sodium phosphate pH 7, was placed in the other syringe. The reaction was started by pneumatic mixing of the two syringes and the release of NTB at 412 nm was measured. The pH of the final reaction mixture was routinely checked.

### **2.5.3 Inactivation of mGSTP1-1 and mutants by hydrogen peroxide**

Time-dependent inactivation was monitored by incubating enzyme with different concentrations of hydrogen peroxide in 100 mM sodium phosphate pH 6.5, in a total volume of 200  $\mu$ l at 30°C. At various intervals aliquots (10  $\mu$ l) of the reaction mix were removed and the remaining GST activity was measured in a final volume of 2 ml (see Section 2.4.1). For each incubation, the enzyme activity was measured over a series of time points and compared to activity at time zero. Samples of the inactivated protein were precipitated using TCA (see Section 2.3.1), and the protein was then run on SDS-polyacrylamide gel as described in Section 2.3.1 except that no  $\beta$ -mercaptoethanol was added to the sample buffer.

## 2.6 Bacterial strains, plasmid vectors and culture media

Culture media (Luria-Bertani broth and Luria-Bertani medium) were made up according to the methods described in Sambrook *et al* (1989). Autoclaving was carried out at 121°C at 1.13 bars for 20 min. Selective components were added to the LB after autoclaving as required. Stock solutions of ampicillin (100 mg/ml), lysozyme (10 mg/ml) and IPTG (1 M) were filter-sterilized with a 0.22 µm sterile filter and stored at -20°C. Bacteria were re-plated from single colonies every 4 weeks and stocks were maintained at -70°C in 15% (v/v) glycerol. The genotypes of the *E. coli* storage and expression strains are listed in Table 2.2.

The vector pET 11a was the vector used for expression of mGSTP1-1 and its mutants. *E. coli* TG1 or *E. coli* DH5α were used routinely for the growth and transformation of plasmids. The BL21(DE3) strain of *E. coli* was used for the expression of proteins by the pET vector family, because it contains an inducible T7 RNA polymerase. *E. coli* BL21(DE3) has a low transformation efficiency and is known to rearrange plasmids. Therefore, genetic manipulation steps on pET vectors were carried out using the strain DH5α. pET vectors used for expression were purified from DH5α, transformed in BL21(DE3) (see Section 2.8.3) and used for protein expression within 1 week of plating out, or were grown overnight and frozen at -70°C in 15% (v/v) glycerol. When required, the bacteria were streaked out on agar plates and incubated overnight at 37°C.



**Table 2.2      Genotypes of *E. coli* strains used in recombinant DNA experiments**

| Strain   | Genotype                                                                               |
|----------|----------------------------------------------------------------------------------------|
| BL21(DE) | <i>hsdS gal (lclts857 ind1 Sam7</i><br><i>Nin5 lacUV5-T7 gene 1)</i>                   |
| TG1      | <i>supE hsdΔ5 thi Δ (lac-proAB)</i><br><i>F[traD36 proAB+ lacIq lacZΔM15]</i>          |
| DH5α     | <i>supE44 ΔlacU169 (080 lacZΔM15)</i><br><i>Hsd R17 recA1 endA1 gyrΔ96 thi-1 relA1</i> |

## **2.7 DNA Manipulation techniques**

### **2.7.1 Plasmid purification**

Large-scale plasmid preparation was conducted using a Qiagen midi plasmid kit which gave approximately 100 µg of plasmid DNA. Small-scale preparation was performed using a Promega Wizard mini-prep plasmid kit which gave approximately 10 µg of DNA.

### **2.7.2 Electrophoresis of DNA in agarose gels**

Horizontal agarose gel electrophoresis was performed using an ATTO AE-6100 electrophoresis apparatus. The agarose gels were prepared in Tris-acetate buffer (TAE; 0.04 M Tris-acetate, 1 mM EDTA, pH 8) and the agarose concentration ranged from 0.8 to 1.2% (w/v), as required. The sample (50 ng – 1 µg of DNA) was mixed with 0.25 volumes of 5 x loading buffer solution [40% (w/v) sucrose, 0.25% (w/v) bromophenol blue]. Gels were run at a constant voltage of 100 V until the tracker dye had run three quarters of the way down the gel.

Gels were stained for 10 to 20 min in ethidium bromide (0.5 µg/ml) and were rinsed briefly in water. DNA bands were visualized by illumination using an ultraviolet light source (UVP Inc., Transilluminator Model TM-20) and the gel was photographed using a Polaroid DS 34 Direct Screen instant camera.

### **2.7.3 Purification of DNA from Agarose gels**

Restriction-endonuclease-digested DNA fragments were electrophoresed through a 1% agarose gel as described (see Section 2.7.2). The DNA was visualized under ultraviolet light following staining with ethidium bromide. Digested fragments were purified using an agarose gel DNA extraction kit (Boehringer Mannheim). The bands of interest were excised using a sterile razor blade, transferred to pre-weighed reaction tubes and solubilisation buffer was added (300  $\mu$ l for every 100 mg of agarose). A 40  $\mu$ l volume of resuspended silica solution was added to the tube and the mixture was incubated for 10 min at 56°C with intermitting vortex-mixing. The silica beads and bound DNA were pelleted by centrifugation at 12,500 rpm for 30 s. The supernatant was discarded and the DNA-containing matrix was resuspended with 500  $\mu$ l of nucleic acid binding buffer and centrifuged as before. The supernatant was again discarded and the matrix resuspended with 500  $\mu$ l of wash buffer. Having repeated this step once any remaining liquid was removed from the tube, which was then left to dry at room temperature for 15-30 min. The bound DNA was eluted from the matrix with 50  $\mu$ l of sterile distilled water. The mixture was vortex-mixed and incubated at 56°C for 10 min. Following centrifugation for 30 s, the DNA-containing solution was transferred to a new reaction tube for further manipulation.



#### **2.7.4 Purification of DNA fragments using the High Pure PCR Product Purification Kit**

The High Pure PCR Product Purification Kit (Promega) is designed for the direct isolation and concentration of PCR products from amplification reactions. In this case a filter-tube unit incorporating a glass fibre fleece was used as a means of binding DNA fragments and the electrophoresis step was omitted. This kit was also used for the purification of restriction-digested plasmid DNA with high recovery yields.

DNA binding buffer was added directly to PCR or restriction digest reactions at 500 µl for every 100 µl of reaction mixture. The High Pure filter tube was attached to the collection tube and the solution was transferred to the upper reservoir. The mixture was centrifuged at maximum speed in a bench-top microcentrifuge for 30 s and the flow-through was discarded. An equal volume of wash buffer was added to the filter tube and the mixture was centrifuged for 30 s. Wash buffer (200 µl) was added for a second time to ensure optimal purity and the flow-through again was discarded following centrifugation. The filter tube was then attached to a clean 1.5 ml reaction tube and 50 µl of sterile distilled water were added in order to elute the DNA from the glass fibre.

### **2.8 Preparation and Transformation of Competent Cells**

#### **2.8.1 Preparation of Fresh Competent Cells**

A single colony of *E. coli* DH5α was inoculated into 4 ml of LB broth and incubated overnight at 37°C with shaking at 270 rpm. A 1 ml sample of this overnight

culture was transferred to 40 ml of LB broth and incubated for approximately 2.5 h until an  $A_{600}$  of 0.4-0.5 was reached. The cells were then centrifuged at 7000 g for 10 min. The pellet was resuspended in 20 ml of ice-cold 0.3 M  $\text{CaCl}_2$  and left on ice for a further 40 min. Following centrifugation at 4000 rpm in an SS34 rotor using a Sorval 5C plus centrifuge for 10 min the cells were resuspended in 1 ml of 0.3 M  $\text{CaCl}_2$ . DNA samples were added to 100  $\mu\text{l}$  aliquots of the competent cells and the suspension was subjected to heat shock treatment involving incubation for 30 min on ice followed by 2 min at 42°C and finally another 2 min on ice. In order to ensure cell recovery and expression of the gene conferring ampicillin resistance 1 ml of LB broth was added and the cells were incubated at 37°C for 1 h. The cells were then centrifuged briefly and resuspended in 200  $\mu\text{l}$  of LB broth. This suspension was plated out onto LB plates containing 100  $\mu\text{g/ml}$  ampicillin and incubated overnight at 37°C.

### **2.8.2 Preparation of Competent DH5 $\alpha$ Cells for Storage**

A single colony of *E. coli* DH5 $\alpha$  was inoculated into 10 ml of LB broth and incubated overnight at 37°C. A 2 ml sample of overnight culture was transferred to 500ml of LB medium and the growth of the cells at 37°C and 270 rpm was monitored at 600nm. When an absorbance value at 600 nm of 0.5 was reached the cells were chilled on ice and recovered by centrifugation at 2500 g for 20 min at 4°C. The resulting cell pellet was resuspended in 20 ml of ice-cold trituration buffer (100 mM  $\text{CaCl}_2$ , 70 mM  $\text{MgCl}_2$ , 40 mM sodium acetate, pH 7.5) and diluted to a final volume of 500 ml with the same solution. Following a 45 min incubation on ice the suspension was centrifuged at 2000 g for 15 min and the pellet was gently

resuspended in 10-50 ml of trituration buffer. The cells were pooled and sterile glycerol was added to a concentration of 15% (v/v). Aliquots were snap frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  for later use.

### **2.8.3 Transformation of competent *E coli* DH5 $\alpha$**

*E. coli* DH5 $\alpha$  competent cells were defrosted on ice. The plasmid to be transformed was added to an aliquot (200  $\mu\text{l}$ ) of the cells and left on ice for 30 min. They were then heat shocked at  $42^{\circ}\text{C}$  for 2 min followed by 2 min on ice. The cells were then allowed to recover by adding 1 ml of LB and incubating them for 1 h at  $37^{\circ}\text{C}$ . The cells were then plated out onto LB plates that contained ampicillin (100  $\mu\text{g/ml}$ ) and incubated overnight at  $37^{\circ}\text{C}$ .

### **2.8.4 Transformation of Super Competent Bacteria**

NovaBlue Singles Competent Cells (Novagen) were defrosted on ice. The plasmid to be transformed was added to an aliquot (50  $\mu\text{l}$ ) of the cells and left on ice for 30 minutes. The cells were then heat shocked at  $42^{\circ}\text{C}$  for 30 s followed by 2 min on ice. The cells were allowed to recover by adding 250  $\mu\text{l}$  of SOC medium and were incubated for 30 minutes at  $37^{\circ}\text{C}$ . The cells were then plated out onto LB plates that contained ampicillin (100  $\mu\text{g/ml}$ ) and incubated overnight at  $37^{\circ}\text{C}$ .



## 2.9 Site Directed Mutagenesis

Site directed mutagenesis was carried out using the PCR method of Fisher *et al.* (1997). Primers (30-35 bp) were designed with the desired mutation in the centre of the primer, with no more than two base-pair changes. A pair of complimentary primers were made for each mutation required. The reaction was performed with pfu turbo polymerase, which is a proof reading polymerase, to reduce the risk of random mutation. The pET 11a plasmid with mGSTP1-1 gene to be used as target for the PCR was purified as described in Section 2.7.1. The final mixture in each PCR tube and the thermal cycles used are listed in Table 2.3. The number of cycles was reduced from normal PCR to 16 to prevent clonal expansion of any (undesired) second-site mutations. Limited cycling, which would result in reduced product yield, was offset by increasing the starting template concentration. After completion of the PCR, the restriction enzyme Dpn1 was added and the mixture was incubated at 37°C for 1 h. This restriction enzyme recognizes a methylated sequence which will degrade the target plasmid as it is methylated by the bacteria it was grown in and the PCR product is not methylated so does not get degraded. After treatment with Dpn1, 1 µl of the PCR product was transformed into competent cells as described in Section 2.8.4. Positive clones were then streaked out on fresh plates and the plasmids were purified as described in Section 2.7.1 before being sequenced.

## 2.10 Synthesis and Reaction of GSSN

Reduced glutathione (4 mM) was mixed with 5,5'-dithiobis(2-nitrobenzoic acid) (6 mM) in a final volume of 1 ml to form a mixed disulfide GSSN (Figure 2.1).

Table 2.3      PCR reactions

(a)      PCR cycling conditions for each mutant

| Name of product  | Template       | Denaturation   | Annealing      | Extension       | Go to       |
|------------------|----------------|----------------|----------------|-----------------|-------------|
| C14A             | mGSTP pET11a   | 95°C for 1 min | 60°C for 1 min | 68°C for 12 min | Step 2 X 16 |
| C47A             | mGSTP pET11a   | 95°C for 1 min | 55°C for 1 min | 68°C for 12 min | Step 2 X 16 |
| C169A            | mGSTP pET11a   | 95°C for 1 min | 67°C for 1 min | 68°C for 12 min | Step 2 X 16 |
| C14/47A (C169*)  | C14A pET11a    | 95°C for 1 min | 68°C for 1 min | 68°C for 12 min | Step 2 X 16 |
| C14/169A (C47*)  | C14A pET11a    | 95°C for 1 min | 54°C for 1 min | 68°C for 12 min | Step 2 X 16 |
| C47/169A (C14*)  | C47A pET11a    | 95°C for 1 min | 65°C for 1 min | 68°C for 12 min | Step 2 X 16 |
| C14/47/169A (3A) | C14/47A pET11a | 95°C for 1 min | 58°C for 1 min | 68°C for 12 min | Step 2 X 16 |

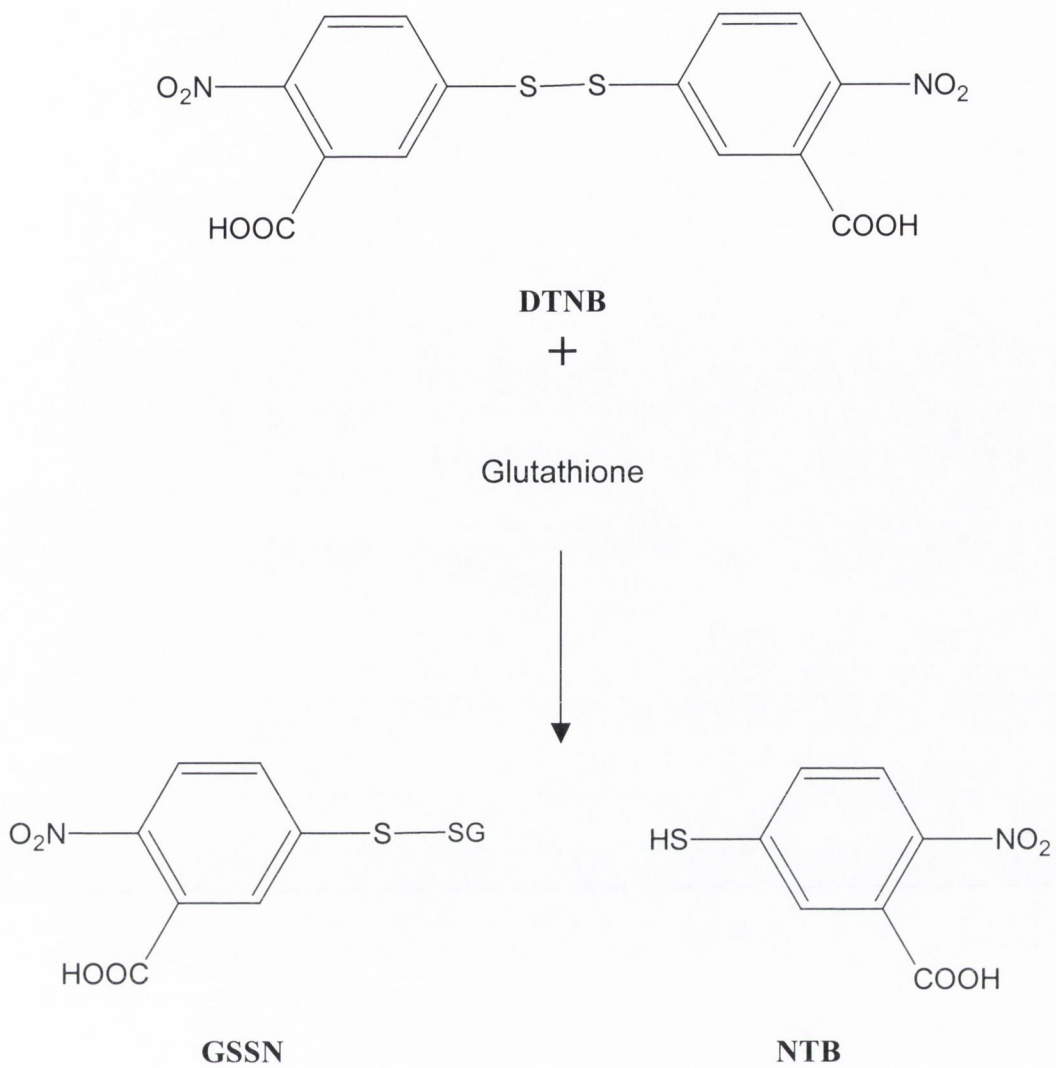
(b)      Components of a typical PCR reaction

|                       |              |
|-----------------------|--------------|
| Template (50 ng)      | 10 µl        |
| Primers (2 pmol/µl)   | 2 µl of each |
| Reaction buffer (10X) | 5 µl         |
| dNTPs (2 mM each)     | 4 µl         |
| Nuclease free water   | 29 µl        |
| Pfu turbo polymerase  | 1 µl         |

The reaction was monitored by measuring the release of NTB (nitrothiobenzoate) at 412 nm in a Philips PU8625 spectrophotometer. The GSSN was purified using DEAE-cellulose chromatography. The GSH/DTNB reaction mixture was applied to a DEAE cellulose column (5 X 1 cm) equilibrated in 10 mM sodium phosphate pH 7.2, and was eluted with the same buffer.

The GSSN (100  $\mu$ M) was mixed with protein (20  $\mu$ M) and the release of NTB was measured as above until completion of the reaction. The experiment was also carried out using [ $^{35}$ S] labelled GSH which was used to 'spike' the cold GSH. The reaction mixture was then loaded on a Sephadex G-25 column (20 X 1.5 cm) to separate the protein from low-molecular-mass compounds, and 2 ml fractions were collected. The protein was monitored by checking the absorbance at 280 nm and the free NTB was monitored by checking the absorbance at 412 nm. For the reactions using  $^{35}$ S, the samples collected from the Sephadex G-25 column were measured by adding 50  $\mu$ l of each fraction to 1 ml of Ecoscint and measuring the counts in a Packard Bell TRI CARB 2100TR scintillation counter.





**Figure 2.1 Reaction of DTNB with glutathione**

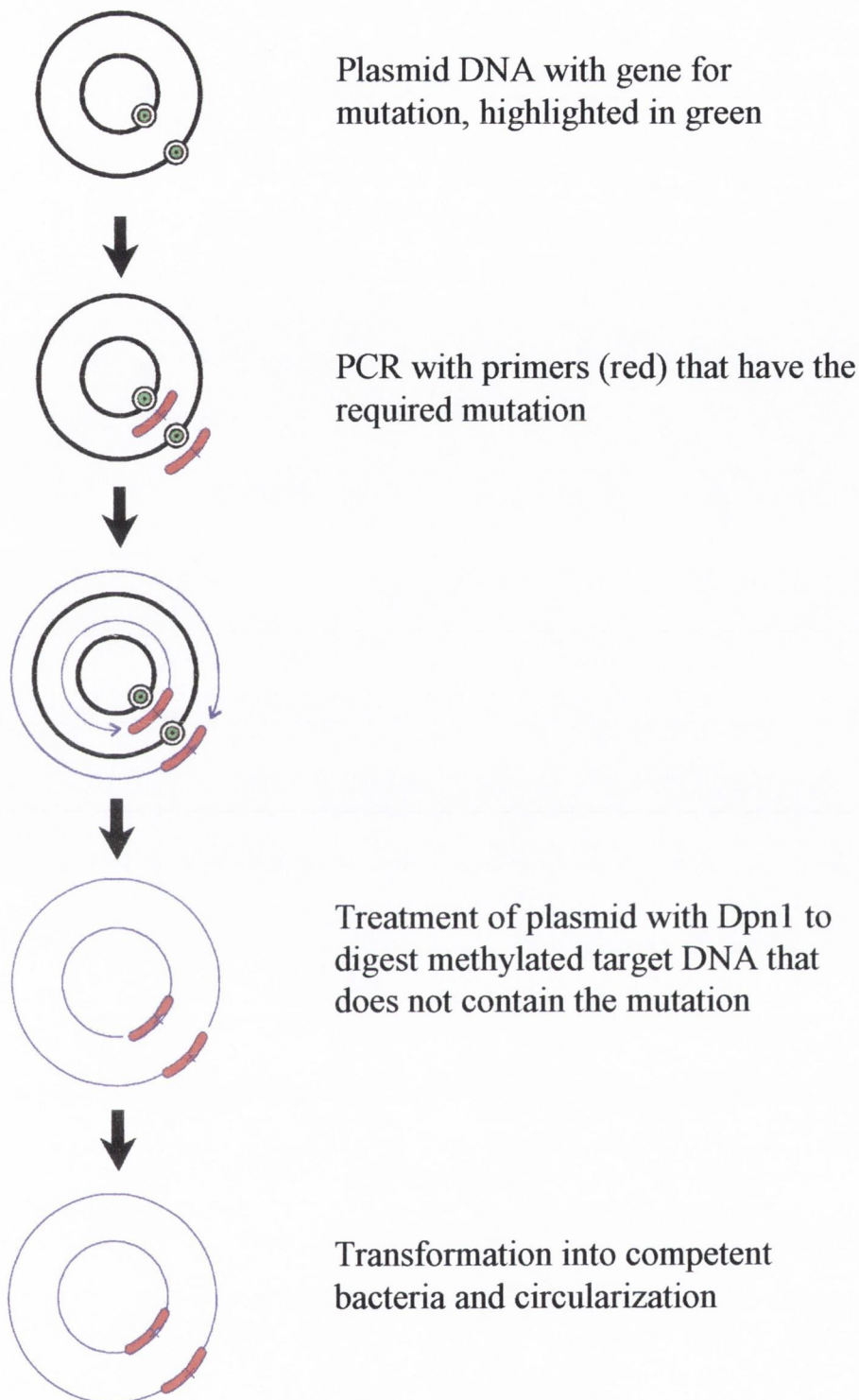
# **Chapter 3**

### 3.1 Introduction to site directed mutagenesis

Results from previous work have shown the importance of cysteine-47 in mGSTP1 as a reactive thiol that may play an important role in the regulation of the enzyme's function. There are two other cysteine residues in mGSTP1, cysteine-14 and cysteine-169. However, their role, if any, on the activity of the enzyme has not been studied. It was therefore decided to change all three cysteine residues to alanine to allow for an in-depth study of their effects on the enzyme.

The method of site-directed mutagenesis adopted was based on that of Fisher *et al.* (1997), which involves the use of PCR to make the mutants. The target for the PCR is the plasmid with the gene for the mutation in it. Primers are designed that contain the required mutation, forward and reverse primers are complimentary and about 30 base pairs long, with the mutation near the middle. A proof-reading polymerase is used to make sure that no other mutations other than the required one are introduced into the plasmid. A low number of cycles (16) is used as a further precaution against unwanted mutations. Upon completion of the PCR, the restriction enzyme Dpn1 is added to the sample to digest methylated DNA. The plasmid used as target is obtained from bacteria that will have methylated the DNA, whereas the plasmid that has been made by PCR has no methylation and is therefore not digested. After the sample has been left to incubate for an hour with 1 unit of Dpn1, 1  $\mu$ l of the PCR reaction is used to transform competent *E. coli* where the plasmid is circularized by endogenous ligases. The *E. coli* is then selected for on LB plates with the relevant antibiotic, (Scheme 3.1).





**Scheme 3.1 Site-directed mutagenesis**

## **3.2 Site directed mutagenesis using polymerase chain reaction (PCR)**

### **3.2.1 Design of primers**

The pET11a-mGSTP1 vector used as the template for PCR was a gift from Dr. T. Bammler, University of Washington, USA. The empty vector is 5.7 kb and the insert is 650 bp long. The vector is ampicillin resistant and the cDNA for mGSTP1 is cloned into the BamH1 and Nde1 sites.

Primers were designed for each of the cysteine to alanine mutations. The sequences of these primers, named for each mutant (C14A, C47A and C169A) are shown in Table 1. The C47A primers have a Bsg1 restriction site designed into them, there was no change of sequence in the other primers that allowed for insertion of a restriction site.

### **3.2.2 Preparation of a template for the PCR**

The pET11a-mGSTP1 vector was transformed into *E. coli* DH5 $\alpha$  (Section 2.8.3). A large-scale plasmid preparation of the vector was performed (Section 2.7.1). For the PCR reaction, the circular vector was diluted to 5 ng/ $\mu$ l.

| Primers | Sequence (5'-3')                                       |
|---------|--------------------------------------------------------|
| C14A1   | CCCAGTTCGAGGGCGGGCTGAGGCCATGCGAATGC                    |
| C14A2   | GCATTCGCATGGCCTCAGCCCGCCCTCGAACTGGG                    |
| C47A1   | TGCTCAAGCCCACT <u>Bsgl</u> <u>GCA</u> CTGTATGGGCAGCTCC |
| C47A2   | GGAGCTGCCCATACAG <u>Bsgl</u> <u>TGC</u> AGTGGGCTTGAGCA |
| C169A1  | CCTGGCCCCTGGCGCTCTGGACAACTTCCCCC                       |
| C169A2  | GGGGGAAGTTGTCCAGAGCGCCAGGGGGCCAGG                      |

Table 3.1 Sequences of oligonucleotides used in site-directed mutagenesis

Sequences of primers used with the codon for alanine are shown in blue. For the C47A1 and C47A2 primers, the inserted restriction site *Bsgl* is underlined.



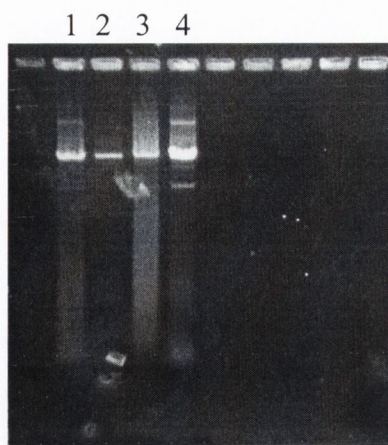
### 3.2.3 PCR reaction

PCR was carried out as described in Section 2.9. The size of the DNA was estimated by comparison with molecular-mass standards. The initial PCR reactions gave multiple bands and conditions of the PCR reactions for each mutant had to be optimized by varying the annealing temperature and addition of DMSO to give a single band of the predicted size (6.3 kb) on agarose gel electrophoresis (Fig 3.1). The first set of PCR reactions used the pET11a-mGSTP1 vector as target to give the single mutants, C14A, C47A and C169A (Fig 3.2). To obtain the double mutants, the target for the PCR was the vectors that had a point mutation. To obtain the C14/47A mutant, the PCR used the C14A vector with primers C47A1 and C47A2. This was repeated to give all three double mutants, and the double mutant C14/169A vector was used with the primers C47A1 and C47A2 to obtain the triple mutant with all three cysteine residues changed to alanine.

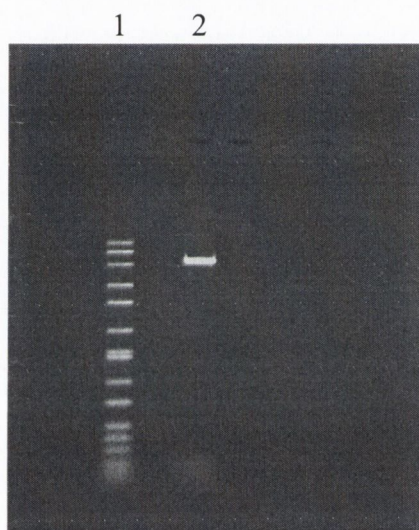
### 3.2.4 Transformation and screening of possible mutants

After the PCR was optimised, a 10 µl sample was run on an agarose gel to show that it contained a single band of the correct size. A 1 µl volume of DpnI was added to the remaining 40 µl of the PCR reaction and left for 1 h at 37°C. From this, a 1 µl sample was transformed into super competent *E. coli*. Transformed *E. coli* colonies were selected on the basis of ampicillin resistance. Possible mutants containing vectors were purified by small scale plasmid purification (Section 2.7.1) and were then sequenced by MWG Biotech. The plasmid containing the possible C47A

a)



b)



**Fig. 3.1 1% Agarose gel of the PCR product for site directed mutagenesis of C47A**

PCR was carried out as described in Section 2.9. The template comprised of 1 ng of mGSTP1-pET11a. Samples of the PCR product (10  $\mu$ l) were separated on 1% agarose gels.

a) Lanes 1-4: PCR with C47A primers, varying the target concentration (10-50 ng).

b) Lane 1: Molecular mass standards (8576, 7427, 6106, 4899, 3639, 2799, 1953, 1882, 1515, 1482, 1164, 992, 710, 492, 359 bp).

Lane 2: PCR of C47A mutant using conditions from Table 2.3.

|       |                   |                    |                   |                    |                   |   |              |
|-------|-------------------|--------------------|-------------------|--------------------|-------------------|---|--------------|
| 1     | <u>ATGCCACCAT</u> | <u>ACACCATTGT</u>  | <u>CTACTTCCCA</u> | <u>GTTGAGGGC</u>   | <u>GGTGTGAGGC</u> |   |              |
|       | M                 | P                  | P                 | Y                  | T                 | I | V            |
|       |                   |                    |                   |                    | Y                 | F | P            |
|       |                   |                    |                   |                    | V                 | R | G            |
|       |                   |                    |                   |                    | R                 | C | E            |
|       |                   |                    |                   |                    |                   | A |              |
|       |                   |                    |                   |                    |                   |   | A (C14A1&2)  |
| (17)  |                   |                    |                   |                    |                   |   |              |
| 51    | <u>CATGCGAATG</u> | <u>CTGCTGGCTG</u>  | <u>ACCAGGGCCA</u> | <u>GAGCTGGAAG</u>  | <u>GAGGAGGTGG</u> |   |              |
|       | M                 | R                  | M                 | L                  | L                 | A | D            |
|       |                   |                    |                   |                    |                   | Q | G            |
|       |                   |                    |                   |                    |                   | Q | S            |
|       |                   |                    |                   |                    |                   | W | K            |
|       |                   |                    |                   |                    |                   | E | E            |
|       |                   |                    |                   |                    |                   | V | V            |
| (34)  |                   |                    |                   |                    |                   |   |              |
| 101   | <u>TTACCATAGA</u> | <u>TACCTGGATG</u>  | <u>CAAGGCTTGC</u> | <u>TCAAGCCCAC</u>  | <u>TTGTCTGTAT</u> |   |              |
|       | T                 | I                  | D                 | T                  | W                 | M | Q            |
|       |                   |                    |                   |                    |                   |   | G            |
|       |                   |                    |                   |                    |                   |   | L            |
|       |                   |                    |                   |                    |                   |   | L            |
|       |                   |                    |                   |                    |                   |   | K            |
|       |                   |                    |                   |                    |                   |   | P            |
|       |                   |                    |                   |                    |                   |   | T            |
|       |                   |                    |                   |                    |                   |   | C            |
|       |                   |                    |                   |                    |                   |   | L            |
|       |                   |                    |                   |                    |                   |   | Y            |
|       |                   |                    |                   |                    |                   |   | A (C47A1&2)  |
| (50)  |                   |                    |                   |                    |                   |   |              |
| 151   | <u>GGGCAGCTCC</u> | <u>CCAAGTTTGA</u>  | <u>GGATGGAGAC</u> | <u>CTCACCTTTT</u>  | <u>ACCAATCTAA</u> |   |              |
|       | G                 | Q                  | L                 | P                  | K                 | F | E            |
|       |                   |                    |                   |                    |                   | D | G            |
|       |                   |                    |                   |                    |                   | D | L            |
|       |                   |                    |                   |                    |                   | T | L            |
|       |                   |                    |                   |                    |                   | Y | Q            |
|       |                   |                    |                   |                    |                   | S | N            |
| (67)  |                   |                    |                   |                    |                   |   |              |
| 201   | <u>TGCCATCTTG</u> | <u>AGACACCTTG</u>  | <u>GCCGCTCTTT</u> | <u>GGGGCTTTTAT</u> | <u>GGGAAAACCC</u> |   |              |
|       | A                 | I                  | L                 | R                  | H                 | L | G            |
|       |                   |                    |                   |                    |                   | R | S            |
|       |                   |                    |                   |                    |                   | L | G            |
|       |                   |                    |                   |                    |                   | L | Y            |
|       |                   |                    |                   |                    |                   | G | K            |
|       |                   |                    |                   |                    |                   | N | Q            |
| (84)  |                   |                    |                   |                    |                   |   |              |
| 251   | <u>AGAGGGAGGC</u> | <u>CGCCCAGATG</u>  | <u>GATATGGTGA</u> | <u>ATGATGGGGT</u>  | <u>GGAGGACCTT</u> |   |              |
|       | R                 | E                  | A                 | A                  | Q                 | M | D            |
|       |                   |                    |                   |                    |                   | M | V            |
|       |                   |                    |                   |                    |                   | N | D            |
|       |                   |                    |                   |                    |                   | G | V            |
|       |                   |                    |                   |                    |                   | E | D            |
|       |                   |                    |                   |                    |                   | L |              |
| (100) |                   |                    |                   |                    |                   |   |              |
| 301   | <u>CGCGGCAAAT</u> | <u>ATGTCACCCT</u>  | <u>CATCTACACC</u> | <u>AACTATGAGA</u>  | <u>ATGGTAAGAA</u> |   |              |
|       | R                 | G                  | K                 | Y                  | V                 | T | L            |
|       |                   |                    |                   |                    |                   | I | Y            |
|       |                   |                    |                   |                    |                   | T | N            |
|       |                   |                    |                   |                    |                   | Y | E            |
|       |                   |                    |                   |                    |                   | N | G            |
|       |                   |                    |                   |                    |                   | K | N            |
| (117) |                   |                    |                   |                    |                   |   |              |
| 351   | <u>TGACTACGTG</u> | <u>AAGGCCCTGC</u>  | <u>CTGGGCATCT</u> | <u>GAAGCCTTTT</u>  | <u>GAGACCCTGC</u> |   |              |
|       | D                 | Y                  | V                 | K                  | A                 | L | P            |
|       |                   |                    |                   |                    |                   | G | H            |
|       |                   |                    |                   |                    |                   | L | K            |
|       |                   |                    |                   |                    |                   | P | F            |
|       |                   |                    |                   |                    |                   | E | T            |
|       |                   |                    |                   |                    |                   | L | L            |
| (134) |                   |                    |                   |                    |                   |   |              |
| 401   | <u>TGTCCCAGAA</u> | <u>CCAGGGAGGC</u>  | <u>AAAGCTTTCA</u> | <u>TCGTGGGTGA</u>  | <u>CCAGATCTCC</u> |   |              |
|       | S                 | Q                  | N                 | Q                  | G                 | G | K            |
|       |                   |                    |                   |                    |                   |   | A            |
|       |                   |                    |                   |                    |                   |   | F            |
|       |                   |                    |                   |                    |                   |   | I            |
|       |                   |                    |                   |                    |                   |   | V            |
|       |                   |                    |                   |                    |                   |   | G            |
|       |                   |                    |                   |                    |                   |   | D            |
|       |                   |                    |                   |                    |                   |   | Q            |
|       |                   |                    |                   |                    |                   |   | I            |
|       |                   |                    |                   |                    |                   |   | S            |
| (150) |                   |                    |                   |                    |                   |   |              |
| 451   | <u>TTTGCCGATT</u> | <u>ACAAC TTGCT</u> | <u>GGACCTGCTG</u> | <u>CTGATCCACC</u>  | <u>AAGTCCTGGC</u> |   |              |
|       | F                 | A                  | D                 | Y                  | N                 | L | L            |
|       |                   |                    |                   |                    |                   | D | L            |
|       |                   |                    |                   |                    |                   | L | L            |
|       |                   |                    |                   |                    |                   | L | I            |
|       |                   |                    |                   |                    |                   | H | Q            |
|       |                   |                    |                   |                    |                   | V | L            |
|       |                   |                    |                   |                    |                   | A |              |
| (167) |                   |                    |                   |                    |                   |   |              |
| 501   | <u>CCCTGGCTGC</u> | <u>CTGGACAAC T</u> | <u>TCCCCCTGCT</u> | <u>CTCTGCCTAT</u>  | <u>GTGGCTCGCC</u> |   |              |
|       | P                 | G                  | C                 | L                  | D                 | N | F            |
|       |                   |                    |                   |                    |                   | P | L            |
|       |                   |                    |                   |                    |                   | L | S            |
|       |                   |                    |                   |                    |                   | A | Y            |
|       |                   |                    |                   |                    |                   | V | A            |
|       |                   |                    |                   |                    |                   | R | L            |
|       |                   |                    |                   |                    |                   |   | A (C169A1&2) |
| (184) |                   |                    |                   |                    |                   |   |              |
| 551   | <u>TCAGTGCCCG</u> | <u>GCCCAAGATC</u>  | <u>AAGGCCTTTC</u> | <u>TGTCCTCCCC</u>  | <u>GGAACATGTG</u> |   |              |
|       | S                 | A                  | R                 | P                  | K                 | I | K            |
|       |                   |                    |                   |                    |                   |   | A            |
|       |                   |                    |                   |                    |                   |   | F            |
|       |                   |                    |                   |                    |                   |   | L            |
|       |                   |                    |                   |                    |                   |   | S            |
|       |                   |                    |                   |                    |                   |   | S            |
|       |                   |                    |                   |                    |                   |   | P            |
|       |                   |                    |                   |                    |                   |   | E            |
|       |                   |                    |                   |                    |                   |   | H            |
|       |                   |                    |                   |                    |                   |   | V            |
| (200) |                   |                    |                   |                    |                   |   |              |
| 601   | <u>AACCGTCCCA</u> | <u>TCAATGGCAA</u>  | <u>TGGCAAACAG</u> | <u>TAG</u>         |                   |   |              |
|       | N                 | R                  | P                 | I                  | N                 | G | N            |
|       |                   |                    |                   |                    |                   | G | K            |
|       |                   |                    |                   |                    |                   | Q | *            |
| (210) |                   |                    |                   |                    |                   |   |              |

**Figure 3.2 mGSTP1-1 DNA sequence**  
The cDNA sequence (black) and its protein translation are shown above. The mutated amino acids are shown in blue along with the primers used, which are the sequences underlined, shown in Table 3.1.



mutation of the mGSTP1 gene was digested with restriction enzyme BsgI and was shown to cut (Fig 3.3).

### 3.3 Purification of mGSTP1 mutants

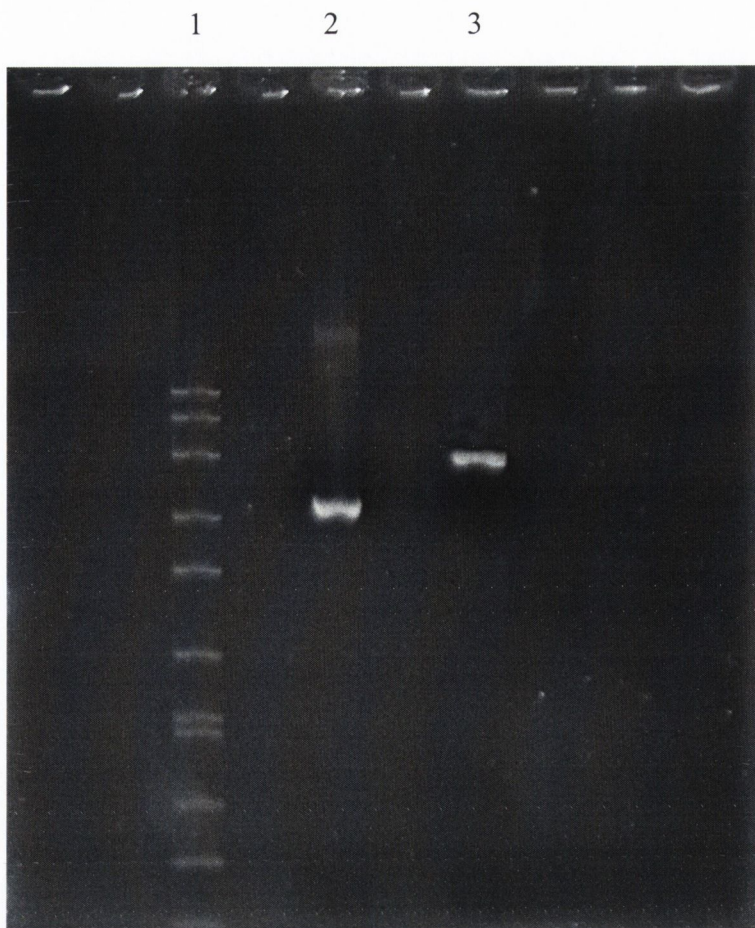
The pET11a plasmids containing the mGSTP1 cDNAs with the various cysteine to alanine mutations were transformed into competent *E. coli* BL21(DE3) cells (Section 2.8.3).

*E. coli* (1 l) for large scale protein purification were cultured as described in Section 2.2.2. The bacteria were lysed by sonication. The GSTs were isolated using glutathione-Sepharose affinity chromatography (10 ml) as described in Section 2.2.2. After elution from the affinity resin, the protein was then gel filtered on Sephadex G-25 to remove the glutathione and was then stored in aliquots at  $-20^{\circ}\text{C}$ .

The yield for the different mutants was similar to that of the native mGSTP1, being between 50 and 80 mg of protein per litre of culture. Fig. 3.4 shows the analysis of the purified proteins by SDS-PAGE. All of the mutants ran as a single band with the same mobility as that of the native mGSTP1. The molecular weight was determined to be 24 kDa by comparison with the molecular-mass standards.

### 3.4 Treatment of mutants with hydrogen peroxide

mGSTP1 and the mutants C14A, C47A, C169A, C169\* and 3A (0.2 mg/ml) were incubated in 100 mM sodium phosphate buffer, pH 8, with 2.5 mM hydrogen peroxide at  $30^{\circ}\text{C}$  for 20 min. The remaining activity was measured (Section 2.4.1) and



**Fig. 3.3 1% Agarose gel of plasmid cut with restriction enzyme BsgI**

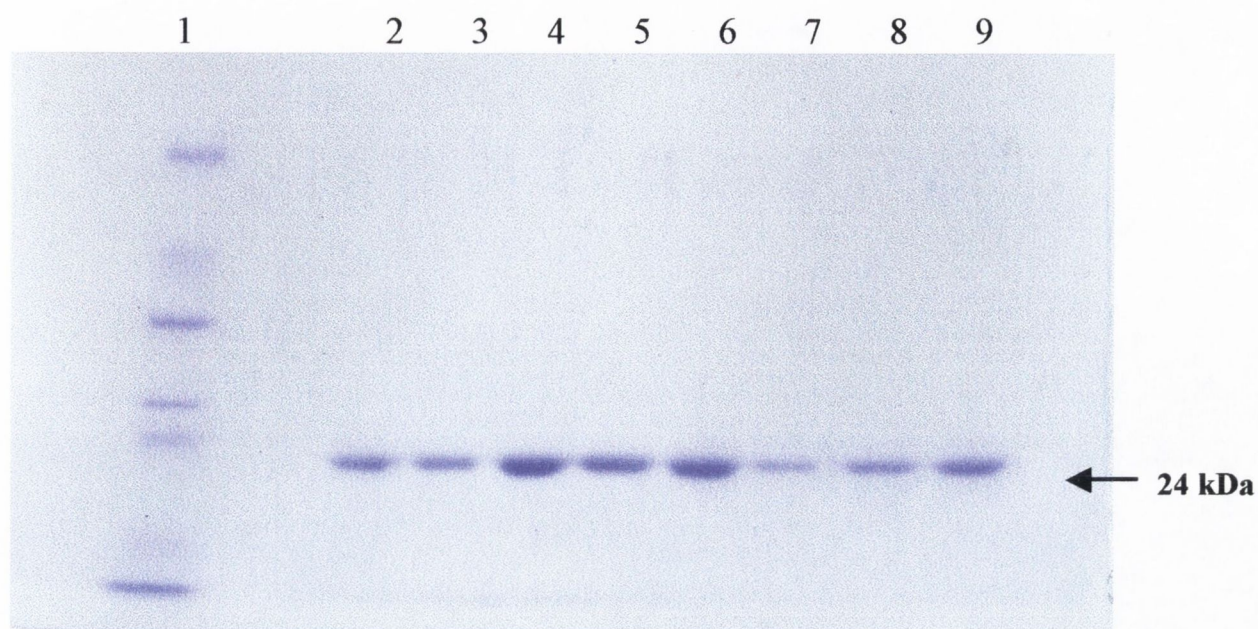
The primers designed to make the C47A mutant of mGSTP1-1 had a BsgI site in them. The plasmid with the suspected C47A mutant was digested with the restriction enzyme BsgI.

Lane 1: Molecular mass standards (8576, 7427, 6106, 4899, 3639, 2799, 1953, 1882, 1515, 1482, 1164, 992, 710, 492, 359 bp).

Lane 2: uncut plasmid of C47A mutant of mGSTP1-1

Lane 3: Plasmid of C47A mutant of mGSTP1-1 digested with BsgI





**Fig 3.4 Analysis of purified mGSTP1 mutants on SDS-polyacrylamide gels**

GSTs were purified by affinity chromatography and were assessed on 12% SDS polyacrylamide gels.

Lane 1; molecular weight standards 2 $\mu$ g of each of the following mixture: bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), and  $\alpha$ -lactalbumin (14.2 kDa).

Lane 2; mGSTP1

Lane 3; C14A-mGSTP1

Lane 4; C47A-mGSTP1

Lane 5; C169A-mGSTP1

Lane 6; C14/47A-mGSTP1

Lane 7; C14/169A-mGSTP1

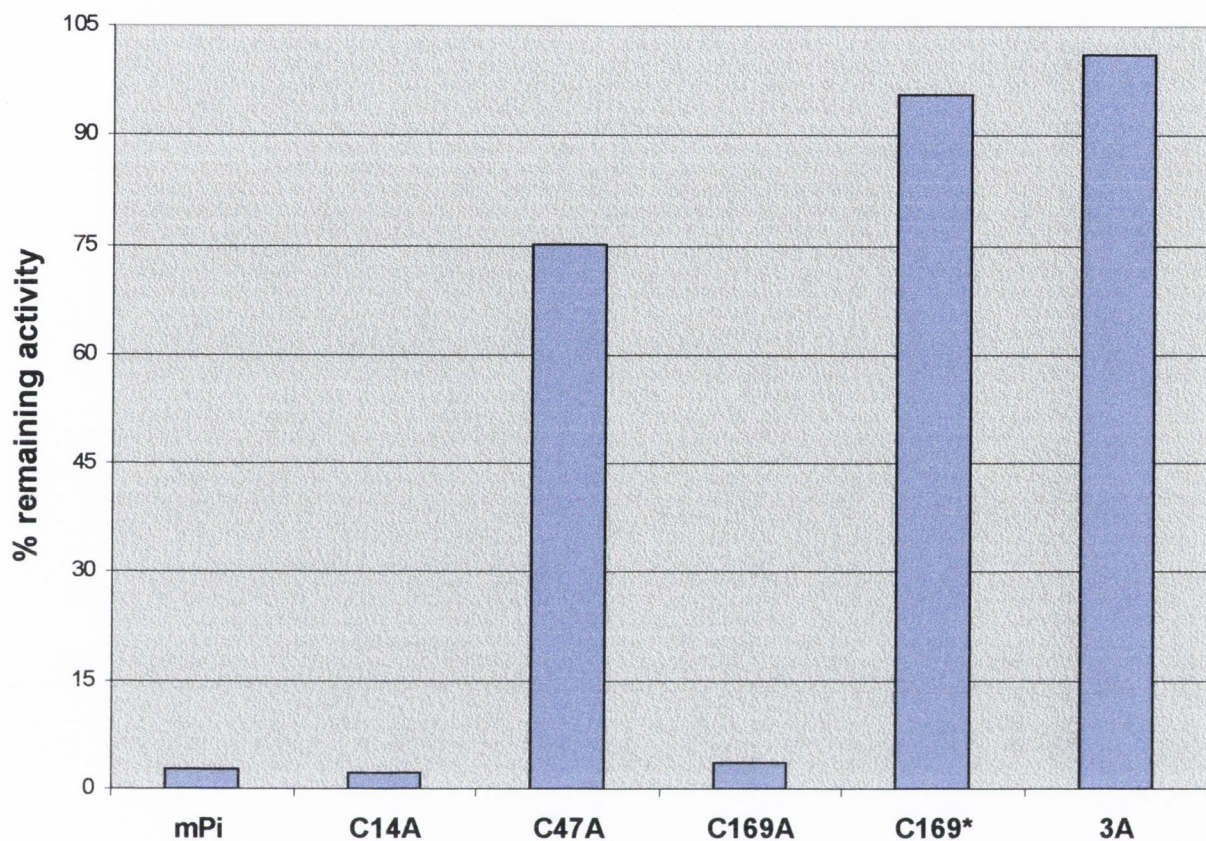
Lane 8; C47/169A-mGSTP1

Lane 9; C14/47/169A-mGSTP1



expressed as a percentage of the original activity. It was found that the activity of the native enzyme decreased to less than 3% of the original activity. The remaining activity of the other mutants was mainly dependent on the presence or absence of C47. The C47A mutant retained 75% of the activity compared to the mutants that contained C47, which, like the native enzyme, were sensitive to  $\text{H}_2\text{O}_2$ . The 3A mutant (with all three cysteines mutated to alanine) retained 100% activity (Fig. 3.5).

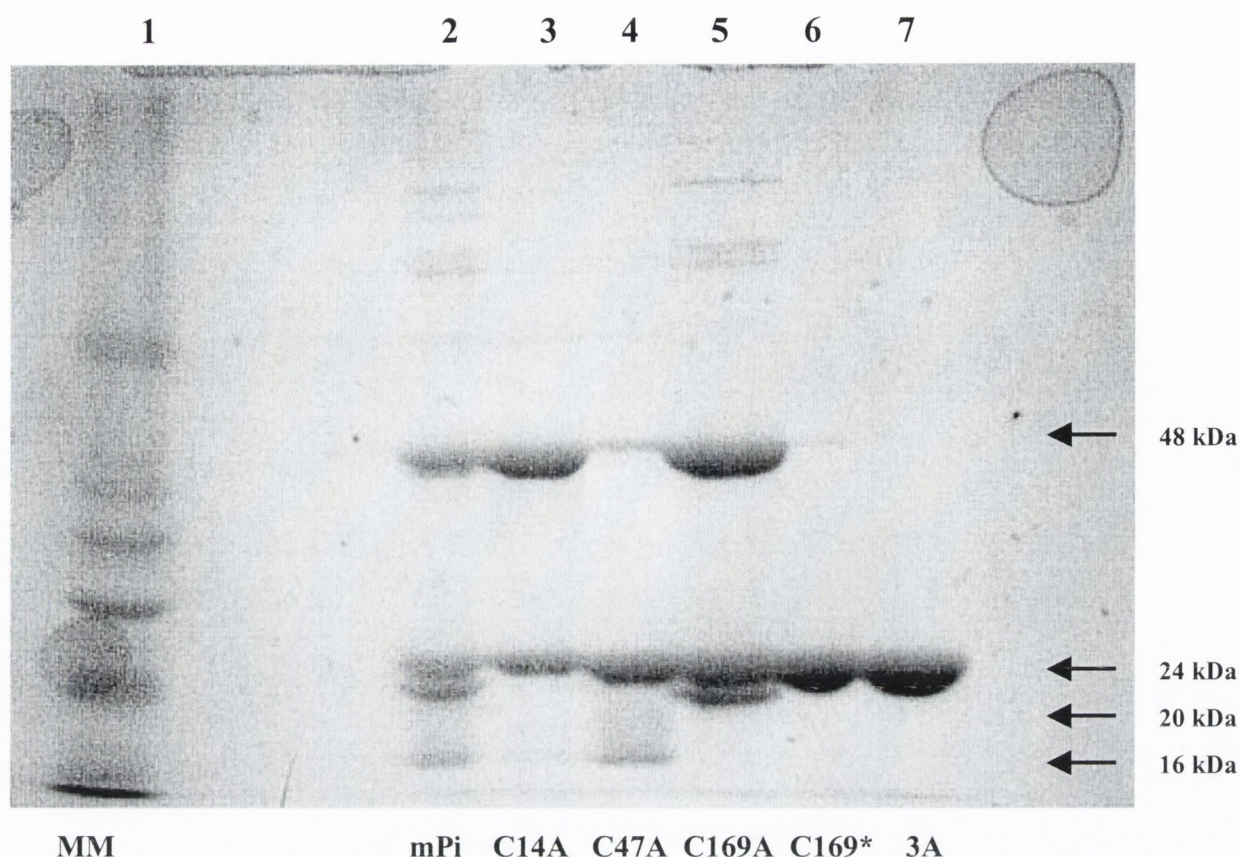
Samples of the above reactions were TCA precipitated and electrophoresed on 15% non-reducing SDS-polyacrylamide gel. A number of multiple bands are clearly seen, including prominent bands at 48 kDa, which, presumably, represent cross-linked dimers. In the case of the wild type enzyme, a band with a mobility corresponding to the unmodified subunit is clearly seen at 24 kDa, together with bands at 20 and 16 kDa. Based on the following analysis (see below), these can be identified as resulting from internal disulfide cross-linking between C14/C47 (20 kDa), C14/C169 (16 kDa) and the non-modified subunit (24 kDa). The C14A and the C169A clearly lack the 16 kDa band, confirming that the C14/C169 cross-linked form is the 16 kDa species while similar arguments for the C169A that the C14/C47 cross-linked form is clearly the 20 kDa form. There is no band on the gel corresponding to a C47/C169 cross-linked form and this may be due to the C47 being much more reactive with itself, to form dimers, giving the 48 kDa band rather than cross-linking with the C169, which is much less reactive as it has a much higher pKa in the native state. The C169\* mutant electrophoreses as a single band with a mobility corresponding to 24 kDa and only a faint band at 48 kDa, showing that the C169 is very unreactive under these conditions. As expected, the 3A mutant has only the one band at 24 kDa as it has no cysteines to take part in cross-linking. Both the native mGSTP1-1 and the C169A



**Fig 3.5 Inactivation of mGSTP1-1 and various cysteine to alanine mutants after incubation with hydrogen peroxide**

mGSTP1-1 and various cysteine to alanine mutants were incubated with 2.5 mM hydrogen peroxide for 20 min and their remaining activity was measured with 1 mM GSH and 1 mM CDNB





**Figure 3.6 Effect of hydrogen peroxide on the mobility of mGSTP1-1 and various cysteine-to-alanine mutants in non-reducing SDS-PAGE**

Samples of protein were treated with hydrogen peroxide as described in Section 3.4 and 15  $\mu$ g were electrophoresed on a non-reducing 15% SDS-polyacrylamide gel.

Lane 1: molecular mass standards: bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and  $\alpha$ -lactalbumin (14.2 kDa).  
 Lane 2: mGSTP1-1; Lane 3: C14A; Lane 4: C47A; Lane 5: C169A; Lane 6: C169\*;  
 Lane 7: 3A mutant.



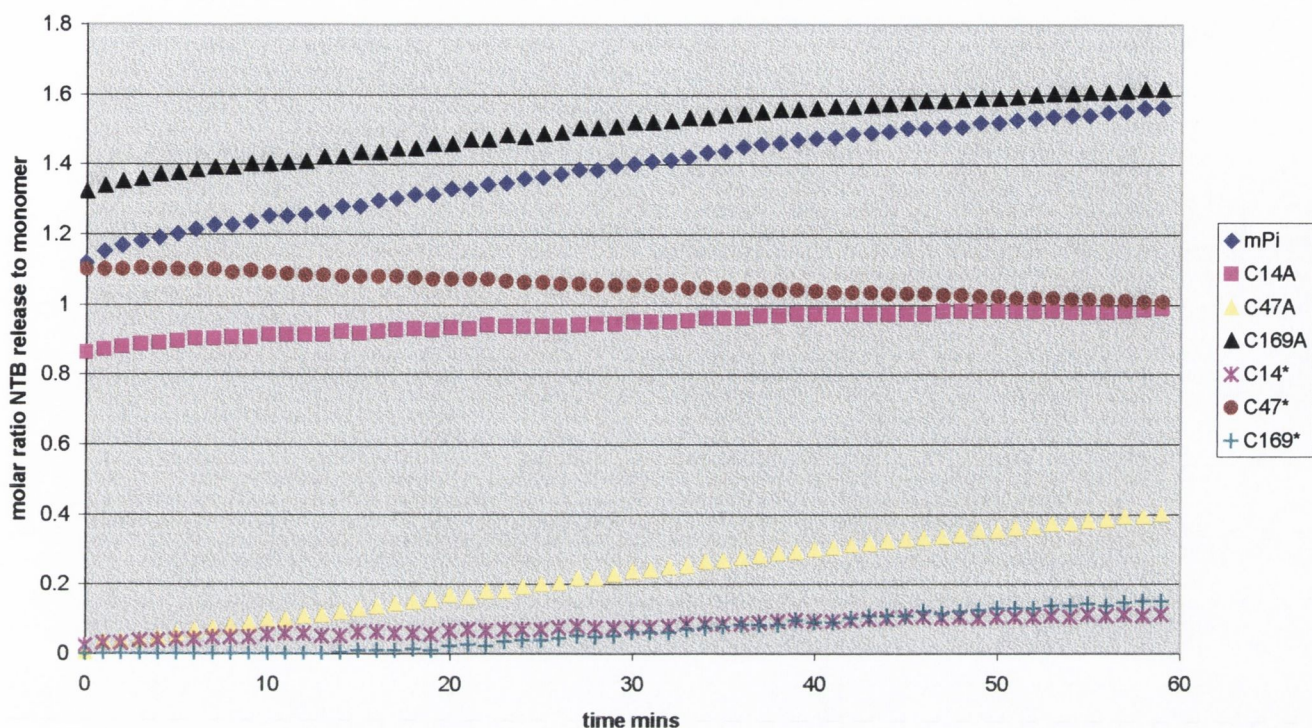
mutant have multiple higher bands, which suggest the formation of multimers that may be dependent on C14 and C47 cross-linking (Fig. 3.6).

### 3.5 Kinetics of reaction of the wild type and mutants with DTNB

The availability of all of the C to A mutants has allowed the definition of cysteine-169 as the “alkaline” thiol, as originally described by Phillips *et al.* (1992). The reaction of the native enzyme and mutants with DTNB at pH 7 is shown in Fig. 3.7. At pH 7, the mutants with only Cys-14 or Cys-169 react with a stoichiometry of less than 0.1 mol NTB released per mol of monomer after a 30 min incubation with a 10 fold excess of DTNB. In contrast, all forms with Cys-47 (native mGSTP1-1, C47\*, C169A and C14A) react immediately with 0.85 – 1.35 mol of NTB released per mol of monomer. The kinetically fast thiol at neutral pH is therefore confirmed as Cys-47.

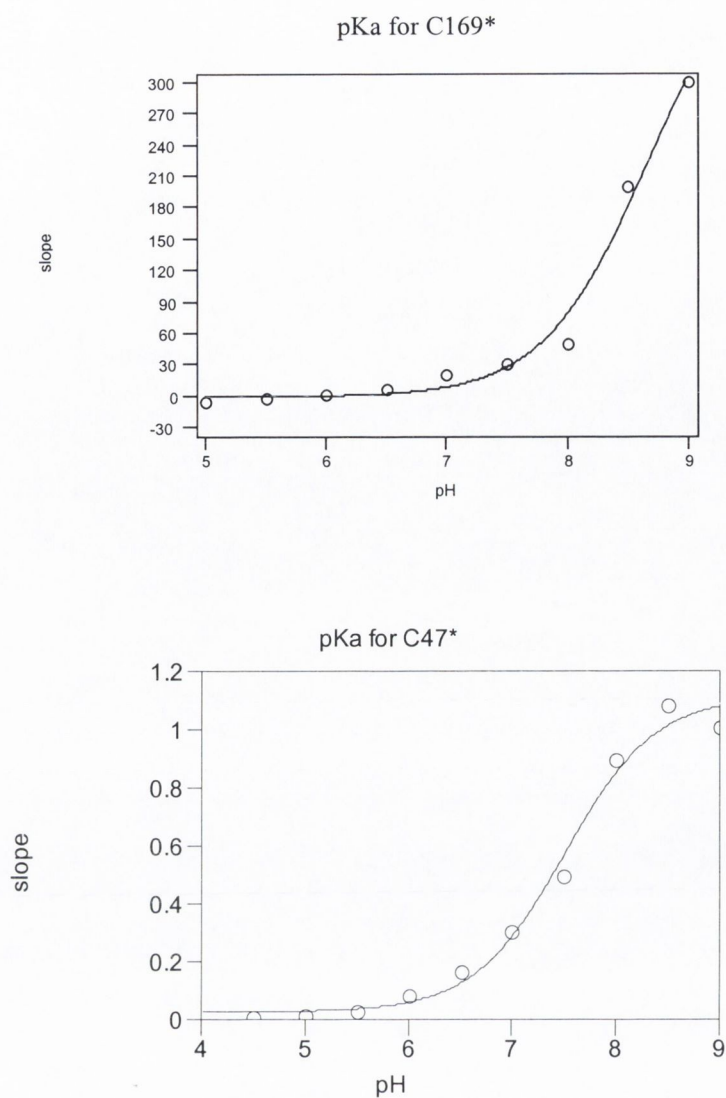
Intriguingly, the native enzyme and the C169A mutant, which have Cys-14 in common, exhibit a further slow reaction at neutral pH, increasing from 1 up to 1.6 mol NTB released per mol of monomer after an incubation of 60 min. The C47\* mutant has 1 mol of reactive thiol but does not show the slow neutral reaction that the Cys-14 containing forms exhibit. When this experiment was repeated with C47\* at a range of pH values, a pKa value of 7.49 was obtained (Fig. 3.8). The C47\* mutant confirms that this is the “neutral”, or low, pKa thiol, while the C169\* mutant reacts with DTNB only at pH values above 8 (see Fig. 3.10). The pKa for the thiol on C169\* is 8.66. It has not been possible to work out a pKa value for Cys-14 as it is buried in the active site and has a very slow reaction with DTNB, even at high pH (see Fig 3.10). A comparison of the mol/mol release of NTB from C14\* and C47\* mutants with that of the C169A mutant, which has both Cys-14 and Cys-47, reveals that there is a significant increase when both cysteines are present on the subunit (Fig. 3.9). This suggests that the reaction of DNTB with Cys-47 somehow makes the Cys-14 more available.

When the reaction of DTNB with GSTP1-1 and the various mutants was carried out with an incubation time of 1 h over a range of pH values to allow for the less reactive thiols, there was a slow reduction in absorbance over time (Fig. 3.10). This trend was checked by repeating the reaction with glutathione instead of protein (Fig.



**Fig 3.7 Incubation of DNTB at pH 7 with mGSTP1-1 and various mutants**

A 20  $\mu\text{M}$  amount of protein was incubated with 200  $\mu\text{M}$  DTNB and the absorbance was measured at 412 nm over a 1 h period. A molar ratio of NTB to mGSTP monomer was calculated taking into account the blank rate of DTNB.

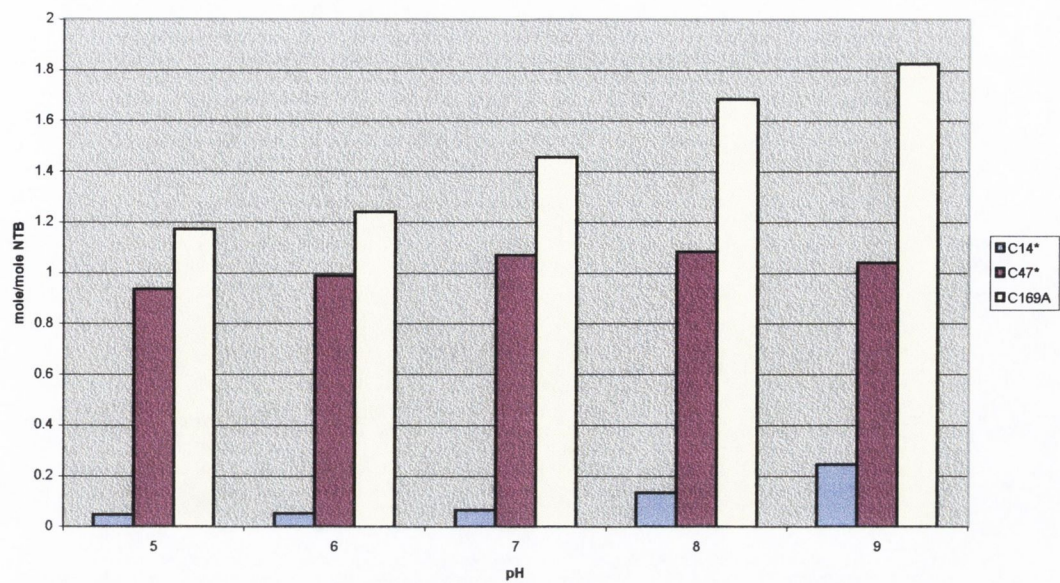


**Figure 3.8 Determination of the pKa for C47\* and C169\* mutants**

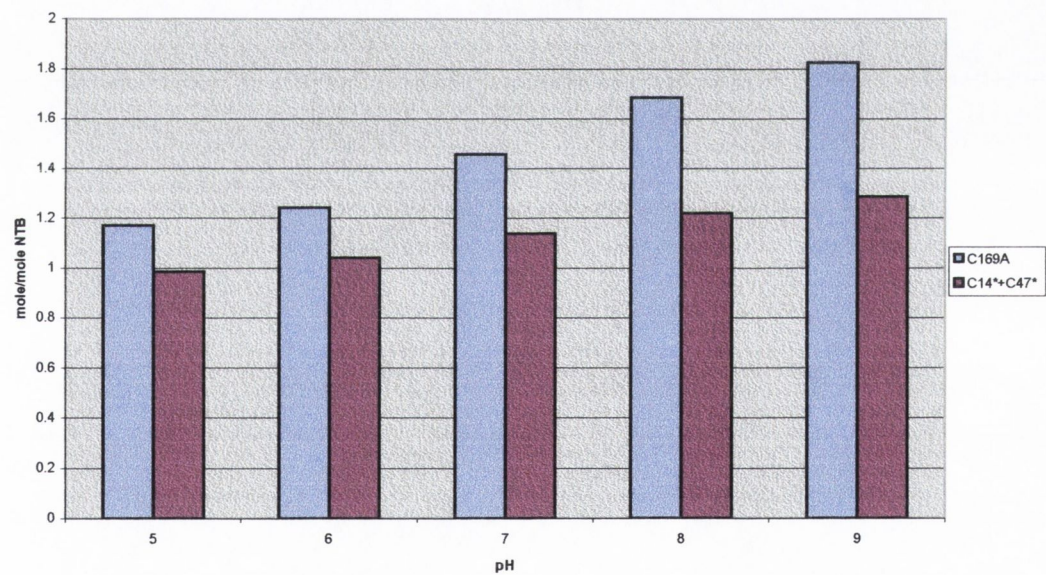
The  $pK_a$  values of C47\* and C169\* were determined by replotting the initial slopes for the reaction of approximately 30  $\mu\text{M}$  protein with 500  $\mu\text{M}$  DTNB at a range of pH values against pH. Data were fitted to the equation:  $F(x) = [a(e^{b(x+c)})-1]/[e^{b(x+c)}+1+d]$



(a)



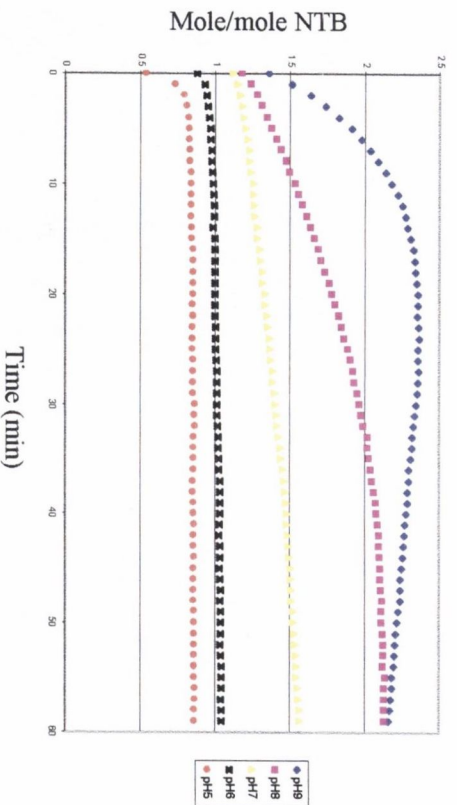
(b)



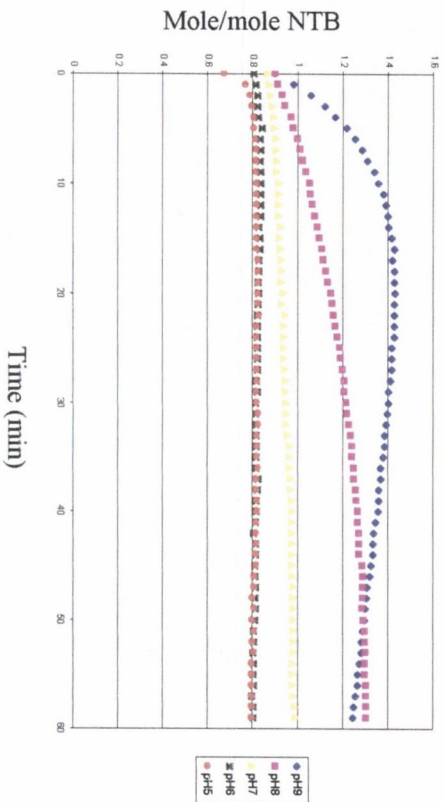
**Figure 3.9 Comparison of Cys-14 with DTNB in the presence and absence of Cys-47**

Comparing NTB release after 20 minutes for a 10 M excess of DTNB at a range of pH values. (a) C14\*, C47\* and C169A mutants shown separately for mol/mol NTB release. (b) The addition of C14\* and C47\* from (a) compared to the C169A mutant, which has Cys-14 and Cys-47 present. This is the representative of two experiments.

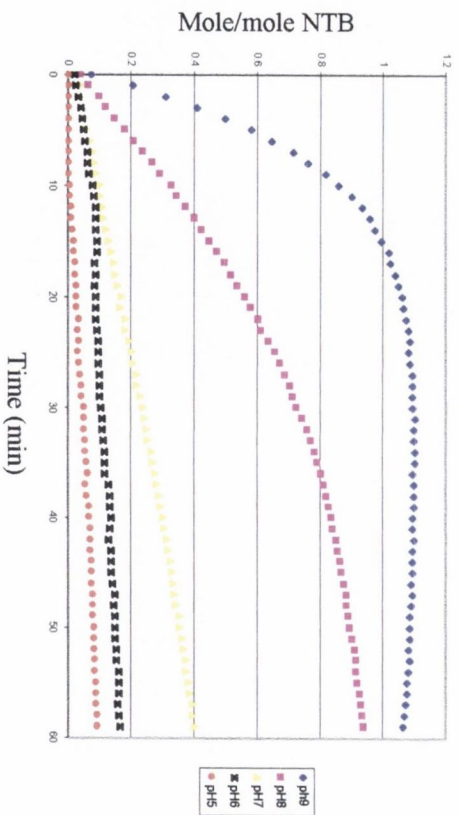
mGSTP1-1



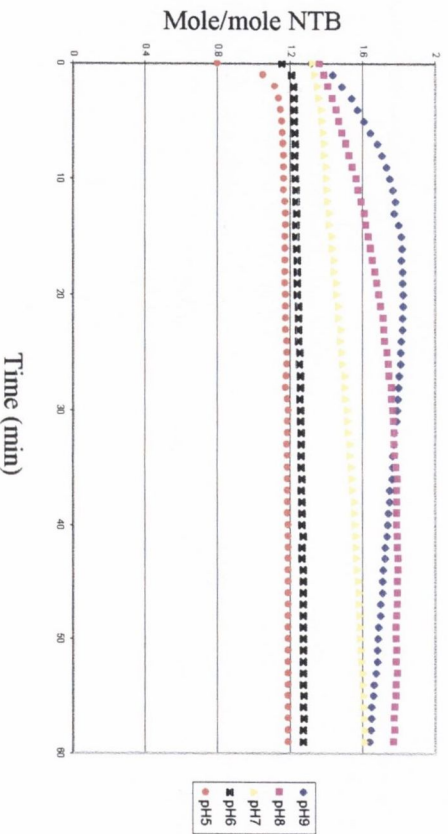
C14A



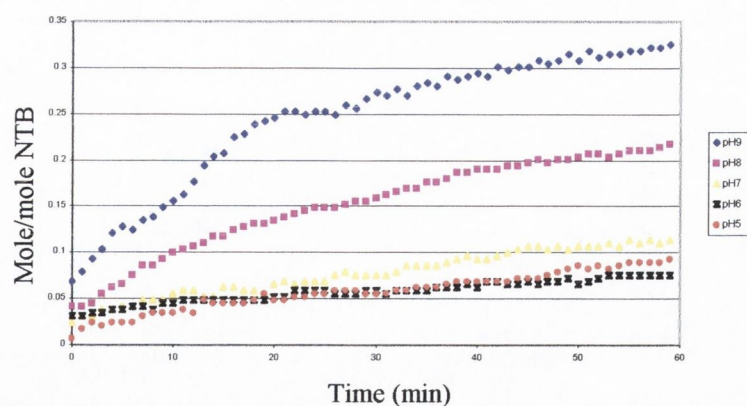
C47A



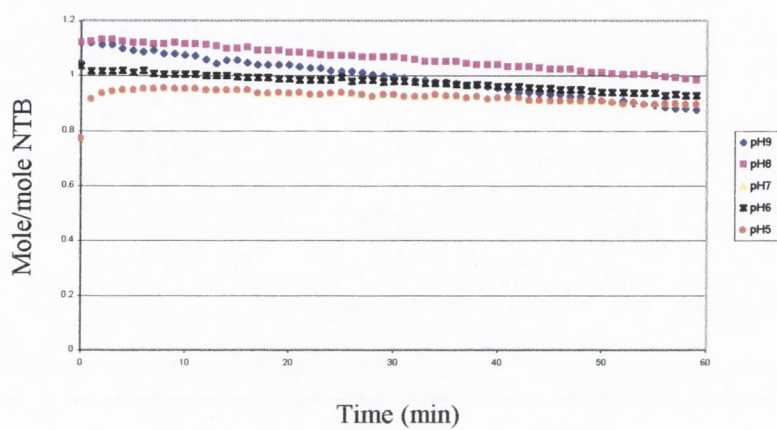
C169A



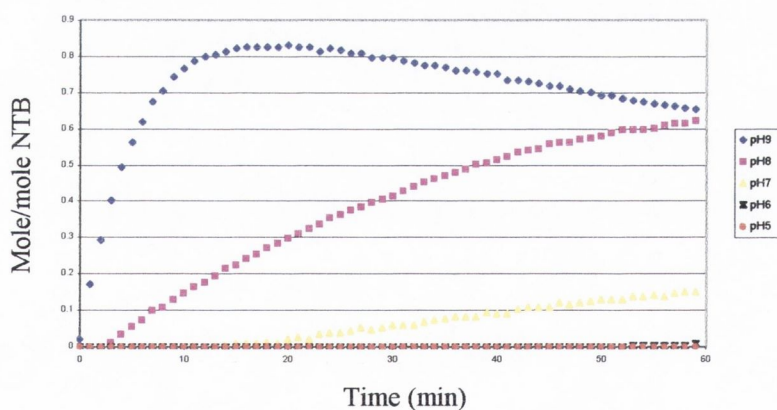
C14\*



C47\*



C169\*



**Figure 3.10 Reaction of mGSTP1-1 and mutants with DTNB over a range of pH values**

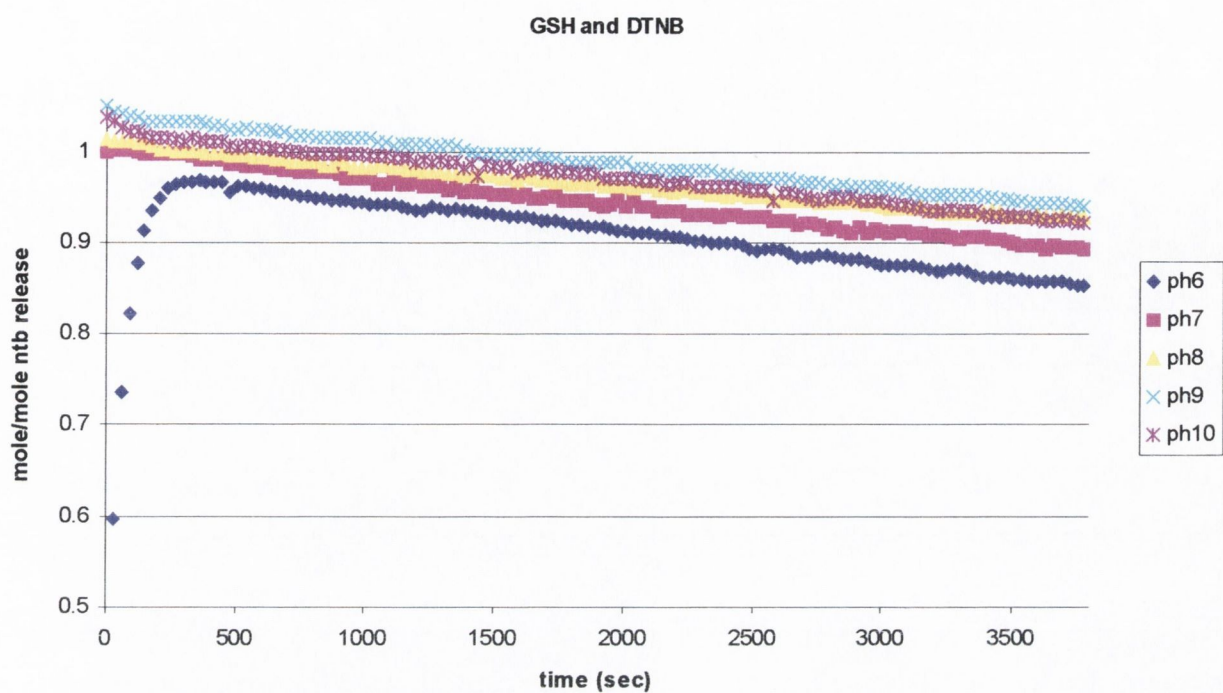
Mol/mol release of NTB was measured at 412 nm over a pH range of 6 to 9 for mGSTP1-1 and the various cysteine to alanine mutants.



3.11) and the same bleaching effect was seen even though the absorbance of DTNB on its own in buffer increases and the blank rate increases as the pH becomes more alkaline. This may lead to complications in working out the exact NTB release of the slow reacting thiols.

### **3.6 CD spectrum of mGSTP1 and mutants**

GSTs are found in high concentrations in many cells and are known to play an albumin-like role in being able to bind non-catalytically to a wide range of endogenous and exogenous ligands, such as bilirubin and steroids. The binding of biliverdin and bilirubin to various serum albumin and biliverdin reductases has been measured using circular dichroism (CD) spectra. Biliverdin and bilirubin in solution have multiple conformations and give no signal when measured using CD, but when they are bound to a protein in a set conformation, they have a distinctive CD spectra that can be measured. This method can also be used for GSTs and was used to measure the binding of biliverdin to mGSTP1-1 and its cysteine to alanine mutants (Table 3.2). The individual cysteine to alanine mutants only give a moderate increase in the  $k_d$  for binding biliverdin but the triple mutant gives nearly a five-fold increase.



**Figure 3.11 Incubation of GSH with DTNB over a range of pH values**

GSH ( $20\ \mu\text{M}$ ) was incubated with  $200\ \mu\text{M}$  DTNB over a pH range of 6 to 10 for 1 h. The graph shows the release of moles of NTB per mol of GSH present, measured at 412 nm. The blank rate was taken into account.

| Protein  | Biliverdin binding Kd ( $\mu\text{M}$ ) |
|----------|-----------------------------------------|
| mGSTP1-1 | $2.96 \pm 0.60$                         |
| C14A     | $4.50 \pm 0.66$                         |
| C47A     | $5.65 \pm 0.66$                         |
| C169A    | $3.908 \pm 0.47$                        |
| 3A       | $14.71 \pm 4.0$                         |

**Table 3.2 Binding of biliverdin to mGSTP1-1 and mutants**



# **Chapter 4**

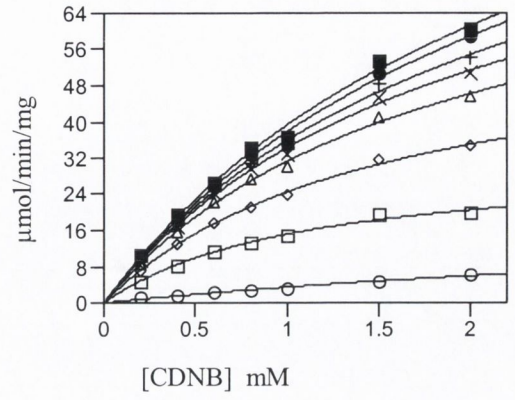
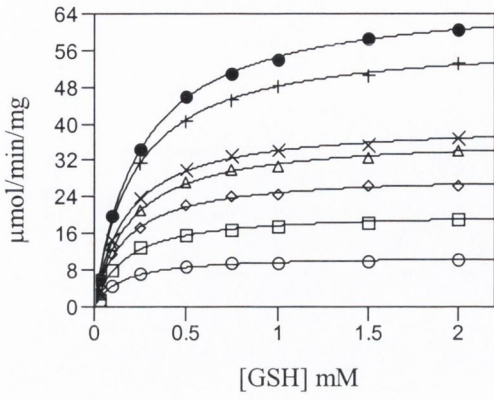
*The data in Chapter 4 are shown to represent the major features described. A complete data set of initial rates in two formats is shown in Appendix I, together with binding data for glutathione and S-(p-nitrobenzyl)-glutathione obtained from measurements of the quenching of protein fluorescence.*

#### **4.1 Kinetic studies of mGSTP1-1 and the various mutants.**

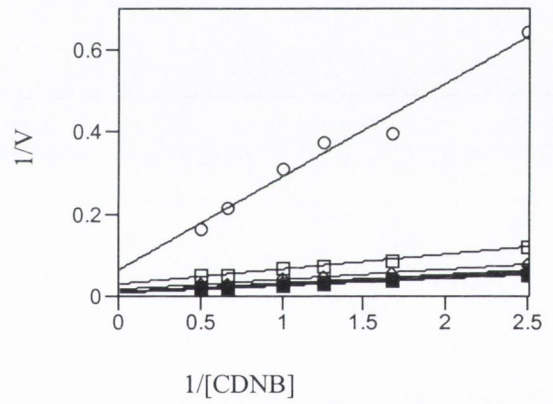
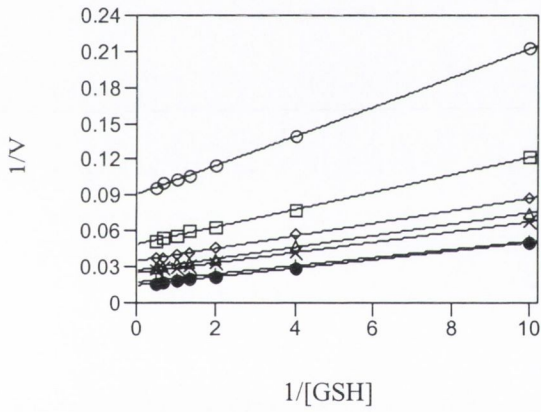
The wild-type native enzyme exhibits Michaelis-Menten initial-rate kinetics consistent with a sequential reaction. An examination of the results obtained with GSH as the variable substrate at fixed levels of CDNB is shown in Fig. 4.1. When plotted as hyperbolae (Fig. 4.1a), the apparent  $V_{\max}/K_m$  (the slope at low substrate concentrations) is clearly dependent on the concentration of CDNB and this is mirrored in the double reciprocal plot (Fig. 4.1b) where the slope (apparent  $K_M/V_{\max}$ , the reciprocal of the apparent  $V_{\max}/K_m$ ) is clearly dependent on the concentration of CDNB. The  $K_m^{\text{GSH}}$ ,  $K_m^{\text{CDNB}}$  and  $V_{\max}$  were calculated from the appropriate slope and intercept replots according to Florini and Vestling (1957) and are shown in Tables 4.1 and 4.2. These kinetic parameters are similar to those obtained by Phillips and Mantle (1992). When the data set for the native enzyme with glutathione as the variable substrate and the CDNB concentration fixed at 1.5 mM, was fitted to the Hill equation, a value for  $n$  of 1.03 was obtained (see Fig 4.2). The native enzyme clearly exhibits no significant co-operativity at high concentrations of CDNB although it should be noted that at low concentrations  $n$  values between 1.1 and 1.2 were obtained.

In contrast all the cysteine to alanine mutants exhibit some degree of cooperativity ( $n>1$ ) when GSH is the variable substrate with the exception of C169A (see Appendix I). When these data sets were fitted to Michaelis-Menten kinetics, a set of apparent kinetic constants was obtained and these were then replotted against

(a)



(b)



**Figure 4.1 Native mGSTP1-1 kinetics**

Kinetics for native mouse GSTP1-1 were performed with glutathione and CDNB as substrates. The results were fitted to a hyperbola (a) and to a double reciprocal plot (b). The kinetics were determined in 0.1 M sodium phosphate pH 6.5, at 30°C. The product was measured spectrophotometrically at 340 nm.



Needs title

|       | GSH          | CDNB         | CDNB(2)      |
|-------|--------------|--------------|--------------|
| mPi   | 168 ± 9.00   | 167 ± 17.07  | 192 ± 28.75  |
| C14A  | 203 ± 21.12  | 195 ± 31.18  | 175 ± 26.35  |
| C47A  | 136 ± 10.02  | 132 ± 62.23  | 134 ± 81.02  |
| C169A | 342 ± 16.62  | 330 ± 78.57  | 342 ± 86.00  |
| C14*  | 169 ± 7.60   | 170 ± 23.65  | 207 ± 35.47  |
| C47*  | 182 ± 17.29  | 188 ± 64.32  | 141 ± 44.43  |
| C169* | 495 ± 145.69 | 693 ± 666.85 | 251 ± 111.47 |
| 3A    | 382 ± 11.20  | 351 ± 41.36  | 331 ± 49.18  |

**Table 4.1**

The true  $V_{\max}$  values were obtained by fitting the primary data sets to a hyperbola to obtain the apparent  $V_{\max}$  values and these were then refitted to a second hyperbola using the fixed concentration as shown. The units for  $V_{\max}$  are  $\mu\text{mol}/\text{min}/\text{mg}$ . CDNB(2) values are taken from replots of Hill plots.

|       | True $K_m$<br>hyperbola |                   | True $K_{0.5}$<br>Hill plots | True $K_m$<br>Double reciprocal plots |           |
|-------|-------------------------|-------------------|------------------------------|---------------------------------------|-----------|
|       | GSH (mM)                | CDNB (mM)         | GSH (mM)                     | GSH (mM)                              | CDNB (mM) |
| mPi   | $0.235 \pm 0.019$       | $2.71 \pm 0.12$   |                              | 0.343                                 | 2.62      |
| C14A  | $0.736 \pm 0.14$        | $5.08 \pm 0.66$   | $0.651 \pm 0.16$             | 1.14                                  | 4.91      |
| C47A  | $1.47^* \pm 0.13$       | $4.91^* \pm 0.31$ | --                           | --                                    | --        |
| C169A | --                      | $5.59 \pm 0.36$   | --                           | --                                    | --        |
| C14*  | $0.79^* \pm 0.13$       | $3.19^* \pm 0.23$ | --                           | --                                    | --        |
| C47*  | $0.85 \pm 0.048$        | $4.39 \pm 0.37$   | $0.557 \pm 0.032$            | 2.76                                  | 4.97      |
| C169* | $12.2 \pm 0.72$         | $6.36 \pm 0.45$   | $14.5 \pm 0.82$              | --                                    | --        |
| 3A    | $5.18 \pm 0.31$         | $5.74 \pm 0.38$   | $5.39 \pm 0.34$              | --                                    | --        |

**Table 4.2  $K_m$  values determined from plots**

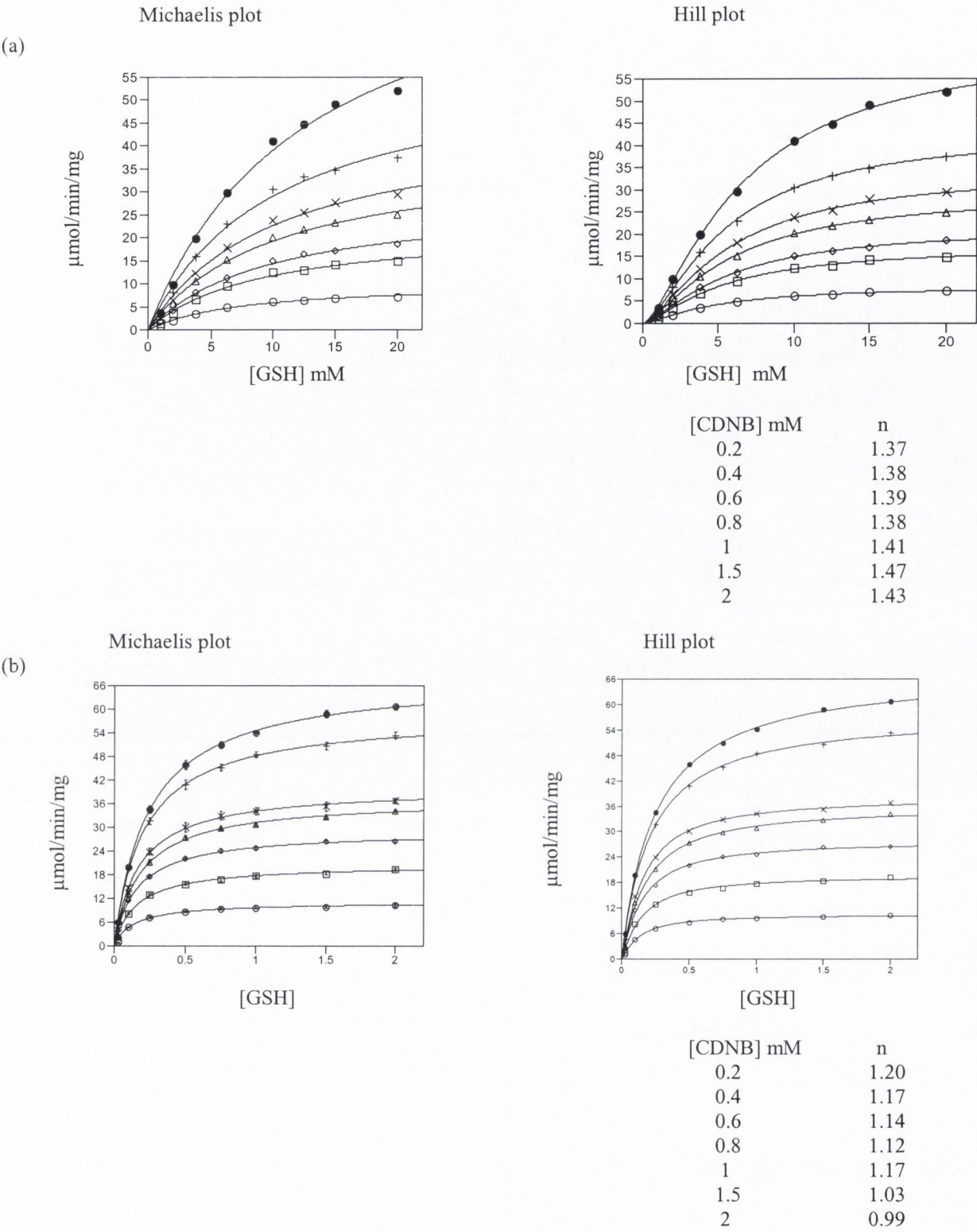
The true  $K_m$  values were calculated by fitting the primary data sets to hyperbolae, the Hill plot and double-reciprocal plots. These were then refitted to a hyperbola or a double reciprocal. Values with \* could not be obtained from a replot and were taken as an average of the apparent  $K_M$  values.

the fixed substrate. The  $K_m$  and  $V_{max}$  obtained from these replots are shown in Tables 4.1 and 4.2. Also shown in Tables 4.1 and 4.2 are the  $K_m$  and  $V_{max}$  values obtained when the data sets were fitted to replots of the apparent kinetic constants from the Hill equation. In each case, by the criteria of least squares, the fit is better to the Hill equation than to a purely Michaelis-Menten mechanism. There is a range of cooperativity for the various mutants with C169\* exhibiting the largest values for the Hill coefficient  $n$  (Table 4.1). The fitting of double reciprocal plots to some data sets of the mutants resulted in kinetic constants that were very inaccurate.

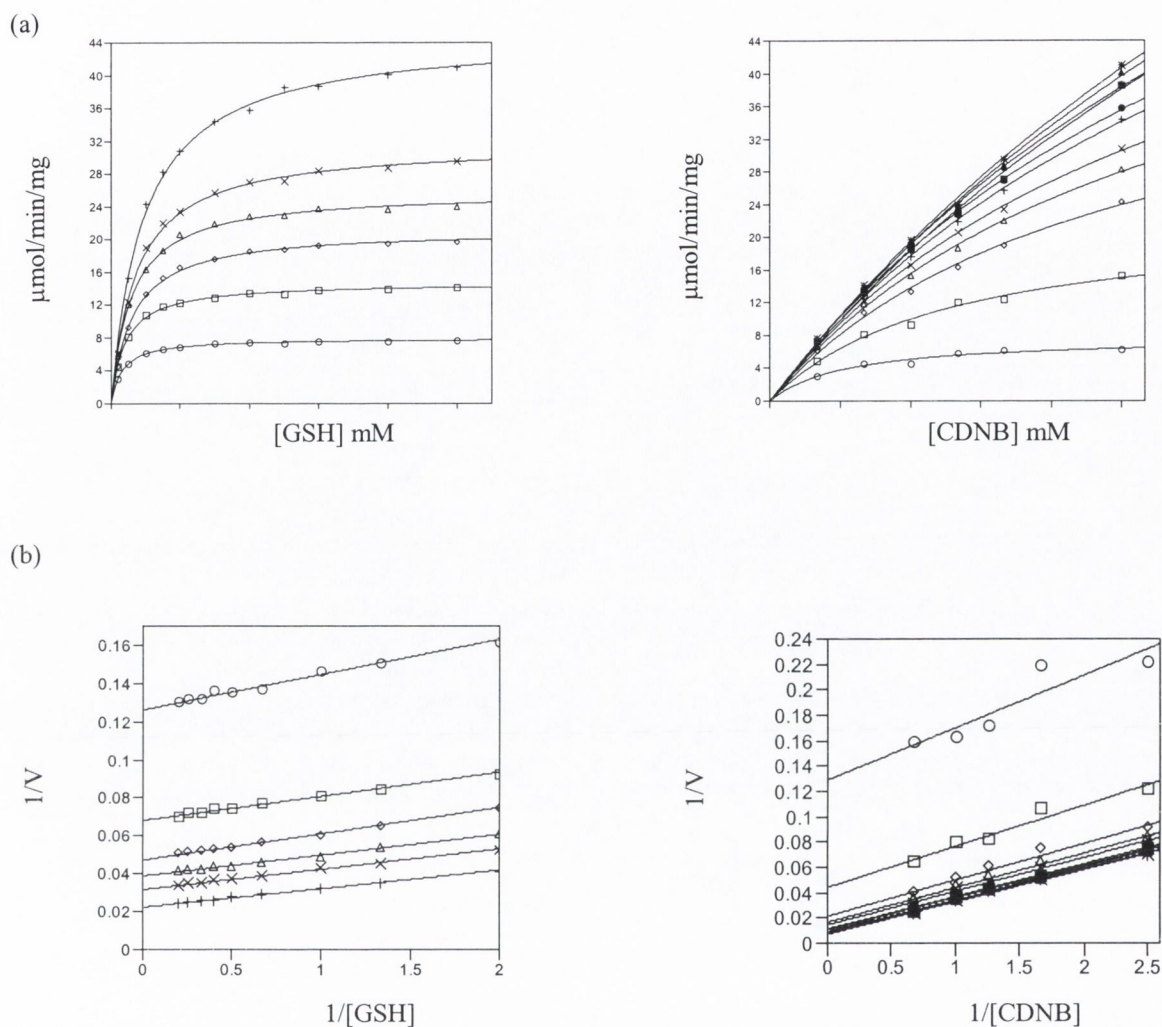
Closer analysis of the initial rate kinetics for two of the mutants, C14A and C169A (Figs. 4.3 and 4.4, respectively) reveals a pattern that is consistent with ping-pong kinetic behaviour. When analysed as hyperbolae (Fig. 4.3a and 4.4a) the apparent  $V_{max}/K_m$  values do not appear to be dependent on the concentration of the fixed substrate and this is clearly seen in double reciprocal plots, which are essentially parallel (Figs. 4.3b and 4.4b). Possible explanations for this unusual behaviour are discussed in Section 6.

Ping-pong kinetics are exhibited by enzymes that follow a reaction mechanism where there is a kinetically significant 'free modified form' of the enzyme, normally denoted  $E'$ . There has to be a clearly defined product-release step, with some element of the substrate left bound to the modified enzyme. Examples include the transfer of reducing equivalents from an amine to the covalently attached FAD of monoamine oxidase, with the liberation of the oxidized imine product or the transfer of amino moieties from a donor amino acid to the enzyme-bound pyridoxal phosphate in various amino transferases (with the release of the first product, the corresponding 2-oxo-acid). Jakoby *et al.* (1974) have suggested that one of the Mu class GSTs exhibits a ping-pong mechanism (see Discussion Chapter 6). However, they argue that CDNB



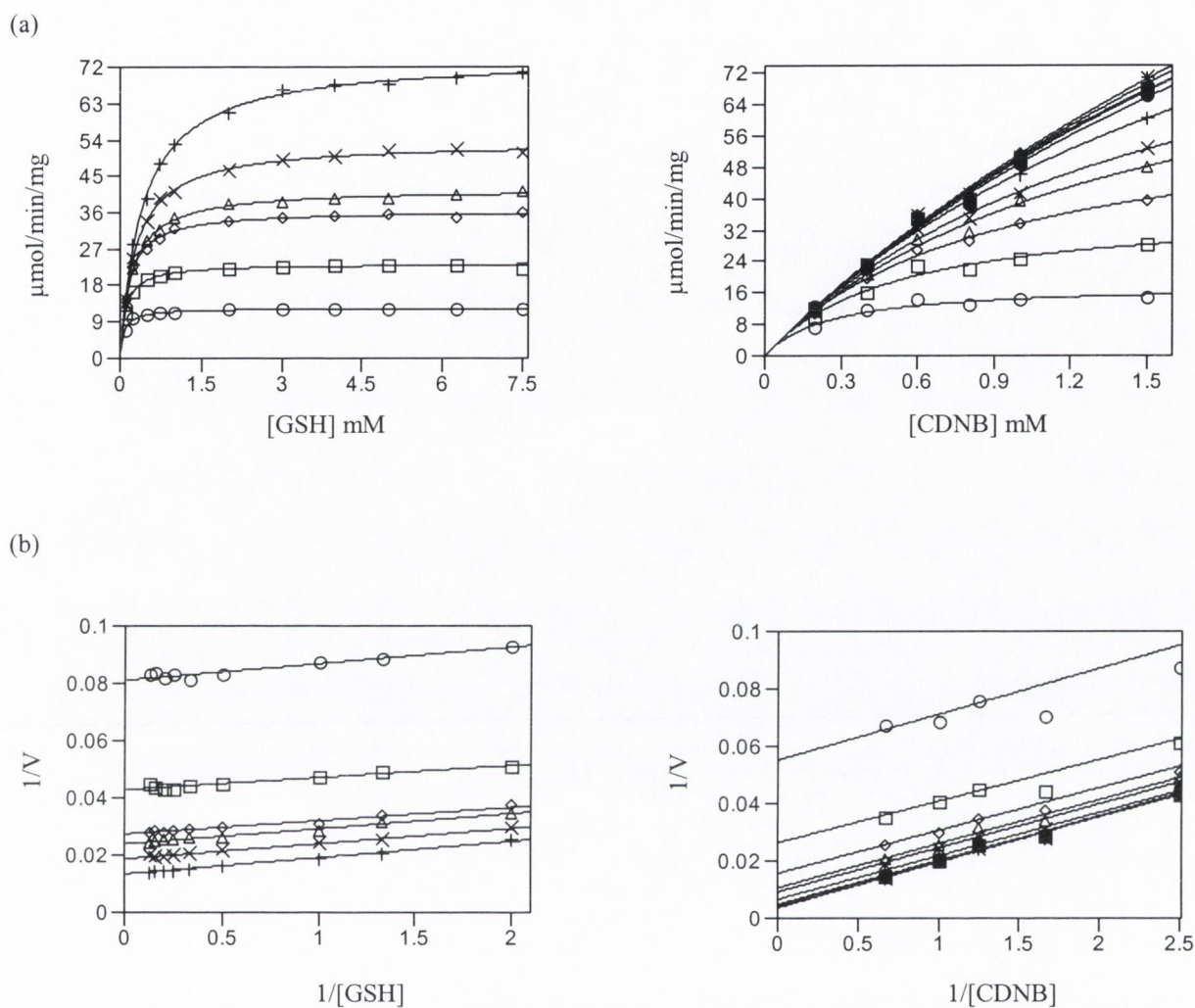


**Figure 4.2 C169\* mutant and mGSTP1-1 native comparison for cooperativity**  
 Kinetics for (a) C169\* and (b) native mouse GSTP1-1 were conducted with glutathione and CDNB as substrates. The kinetics were carried out in 0.1 M sodium phosphate pH 6.5, at 30°C. The product was measured spectrophotometrically at 340 nm. The results were fitted to a hyperbolae and to a Hill plot.



**Figure 4.3 Kinetics of the C14A mutant of mGSTP1-1**

Kinetics for the C14A mutant were determined with glutathione and CDNB as substrates. The results were fitted to a hyperbola (a) and to a double reciprocal plot (b). The kinetics were determined in 0.1 M sodium phosphate pH 6.5, at 30°C. The product was measured spectrophotometrically at 340 nm.



**Figure 4.4 Kinetics of the C169A mutant of mGSTP1-1**

Kinetics for the C169A mutant were determined with glutathione and CDNB as substrates. The results were fitted to a hyperbola (a) and to a double reciprocal plot (b). The kinetics were determined in 0.1 M sodium phosphate pH 6.5, at 30°C. The product was measured spectrophotometrically at 340 nm.

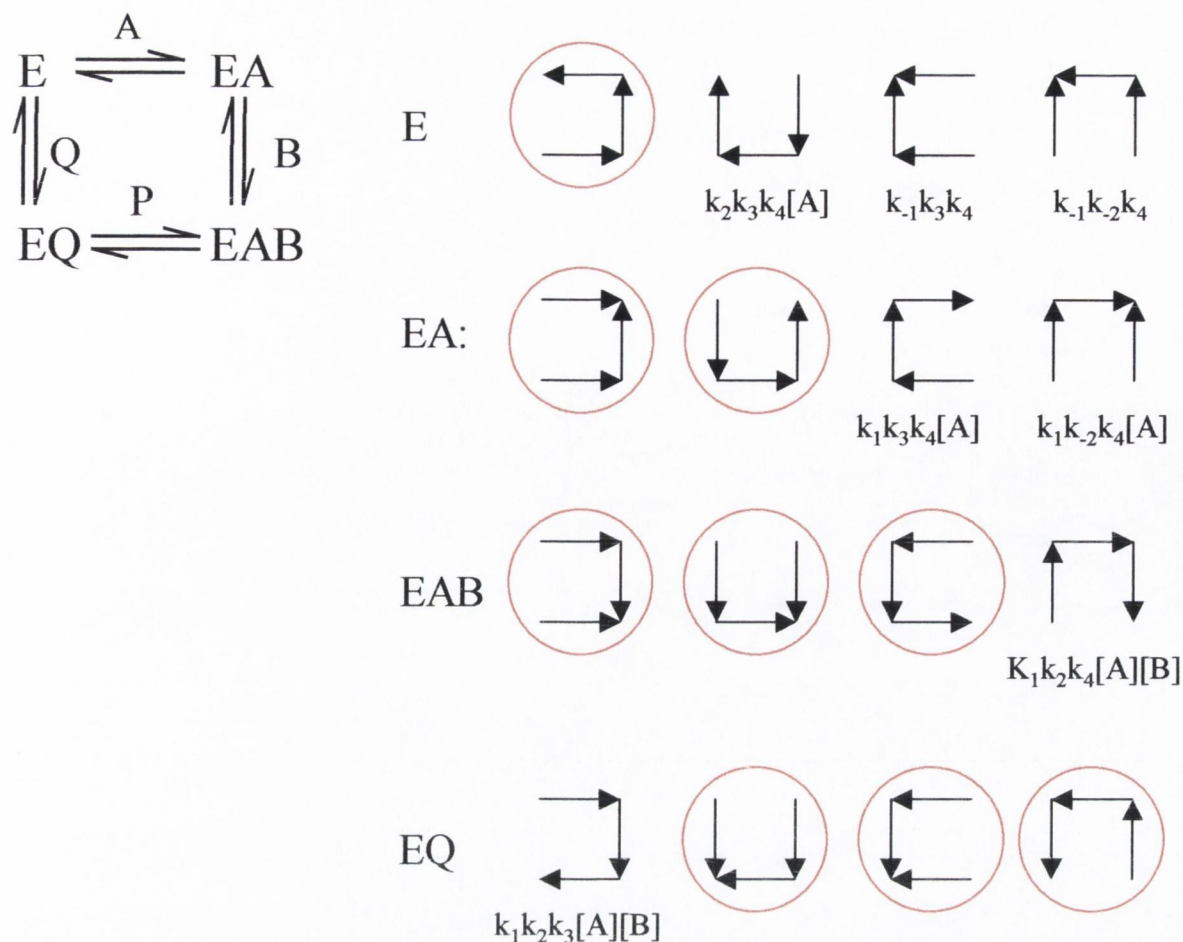


was the leading substrate and that chloride dissociated as the first product while an ill defined ‘dinitrophenyl derivative’ remained bound to the enzyme. For various reasons, discussed more fully in Section 6, it seems more reasonable to argue that the irreversible dissociation of the ‘forgotten’ GST product, the proton, is more likely. Therefore the appropriate rate equations will be developed here to allow their discussion elsewhere.

Shown in Scheme 1 is the classic derivation of an ordered sequential mechanism using the King-Altman procedure (King *et al.*, 1956). Using the arrow patterns with the constraint that no P or Q are added back so that  $P = Q = 0$ , and patterns containing P and/or Q can be deleted (red circles), then the sum of the remaining patterns ( $\Sigma$ ) gives the denominator term. The numerator term is defined as the product of all rate constants ( $n$ ) and associated substrate concentrations in the forward direction so that  $v_o/E_t$  is then easily defined as the ratio  $n[A][B]/\Sigma$  (Eq. 1). By simplifying the kinetic coefficients, as shown in Eq. 2, and then dividing throughout by  $\phi_{AB}$ , we obtain the normal form of the rate equation (Eq. 3). It is the presence of the  $K_{ia}K_B$  term that dictates that non-parallel double reciprocal plots are diagnostic in this case, and this rate equation applies to the native enzyme and most of the mutants studied in this thesis.

However, in the case of the C14A and C169A mutants, I suggest that a simple modification of this mechanism can produce the apparent ping-pong behaviour. As shown in Scheme 2, the modified mechanism introduces an irreversible release of the first product ( $H^+$ ) to form a free modified form  $E.GS^-$  ( $E'$ ). This then binds CDNB and rapidly forms the DPN-G product, which is indicated as EQ. I assume that the release of chloride is irreversible. A fuller version of this mechanism is shown in Scheme 3 as a sequential mechanism to highlight that decoupling of the binding of GSH from the

Scheme 1 is the classic derivation of an ordered sequential mechanism using the King-Altman procedure (King *et al.*, 1956). Using the arrow patterns with the constraint that no P (chloride anion) or Q (DNPG) are added back so that  $P = Q = 0$ , and patterns containing P and/or Q can be deleted (red circles), then the sum of the remaining patterns ( $\Sigma$ ) gives the denominator term. The numerator term is defined as the product of all rate constants ( $n$ ) and associated substrate concentrations in the forward direction so that  $v_o/E_t$  is then easily defined as the ratio  $n[A][B]/\Sigma$  (Eq. 1). By simplifying the kinetic coefficients, as shown in Eq. 2, and then dividing throughout by  $\phi_{AB}$ , we obtain the normal form of the rate equation (Eq. 3). Note that A is GSH, B is CDNB, Q is DNPG and P is chloride anion.



$$\frac{n}{\phi_{AB}} = \frac{k_1k_2k_3k_4}{k_1k_2(k_3+k_4)} = \frac{k_3k_4}{k_3+k_4} = k_{cat}$$

$$\frac{v_o}{E_t} = \frac{(k_1k_2k_3k_4)[A][B]}{k_{-1}k_4(k_3+k_2) + k_1k_4(k_3+k_2)[A] + k_2k_3k_4[B] + k_1k_2(k_3+k_4)[A][B]} \quad \text{Eq. 1}$$

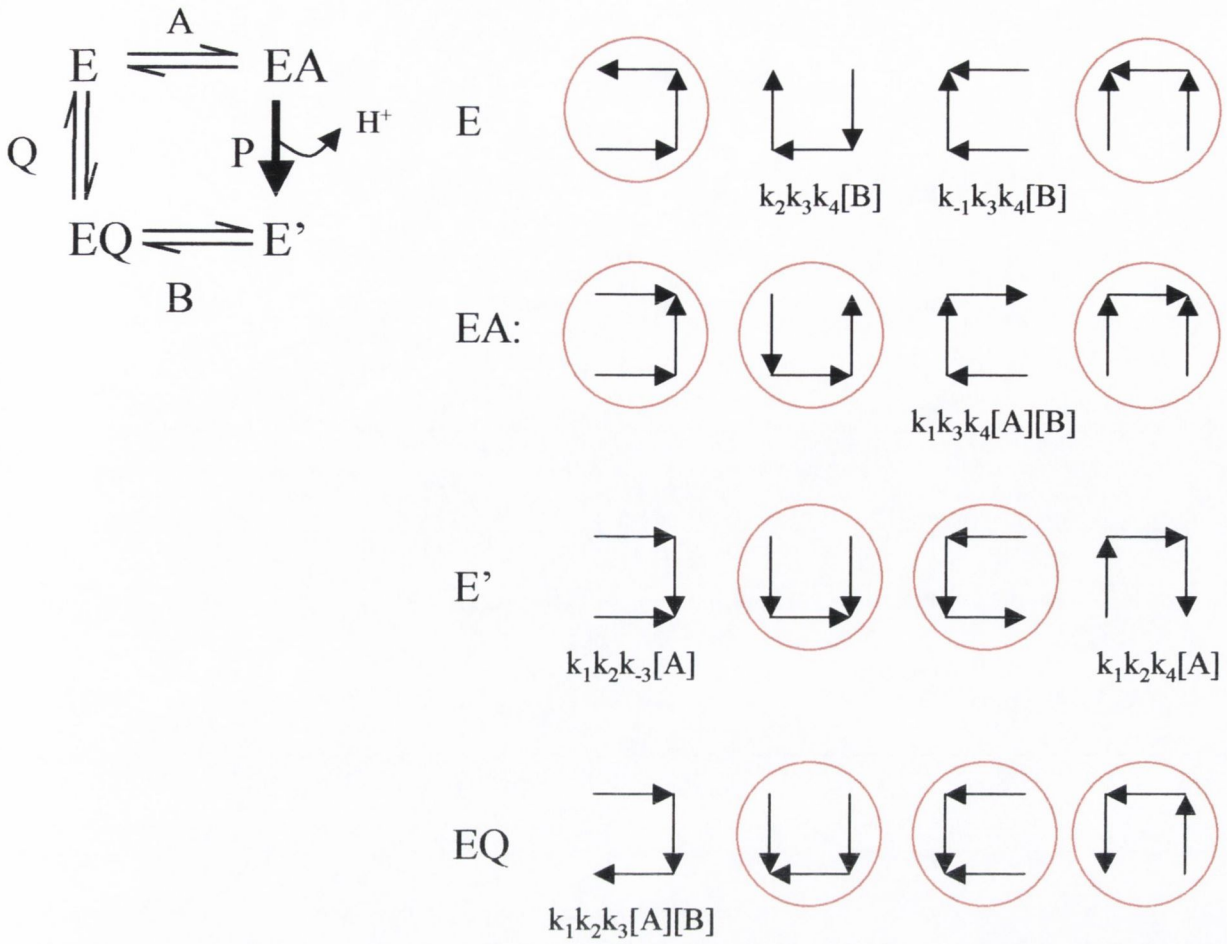
$$\frac{v_o}{E_t} = \frac{n[A][B]}{\phi_0 + \phi_A[A] + \phi_B[B] + \phi_{AB}[A][B]} \quad \text{Eq. 2}$$

$$v_o = \frac{V_{max}[A][B]}{K_{ia}K_B + K_B[A] + K_A[B] + [A][B]} \quad \text{Eq. 3}$$

Scheme 1



Scheme 2 shows the derivation for the ping-pong mechanism for mGSTP1-1 mutants C14A and C169A using the King-Altman procedure (King *et al.*, 1956). Using the arrow patterns with the constraint that no P (chloride anion) or Q (DPNG) are added back so that  $P = Q = 0$ , and patterns containing P and/or Q can be deleted (red circles), then the sum of the remaining patterns ( $\Sigma$ ) gives the denominator term. The numerator term is defined as the product of all rate constants ( $n$ ) and associated substrate concentrations in the forward direction so that  $v_o/E_t$  is then easily defined as the ratio  $n[A][B]/\Sigma$  (Eq. 1). By simplifying the kinetic coefficients, as shown in Eq. 2, and then dividing throughout by  $\phi_{AB}$ , we obtain the normal form of the rate equation (Eq. 3). Note that A is GSH, B is CDNB, Q is DNPG and P is chloride anion.



$$\frac{n}{\phi_{AB}} = \frac{k_1 k_2 k_3 k_4}{k_1 k_3 (k_2 + k_4)} = \frac{k_2 k_4}{k_2 + k_4} = k_{\text{cat}}$$

$$\frac{v_o}{E_t} = \frac{(k_1 k_2 k_3 k_4) [\text{A}] [\text{B}]}{k_1 k_2 (k_{-3} + k_4) [\text{A}] + k_3 k_4 (k_2 + k_{-1}) [\text{B}] + k_1 k_3 (k_2 + k_4) [\text{A}] [\text{B}]}$$

Eq. 1

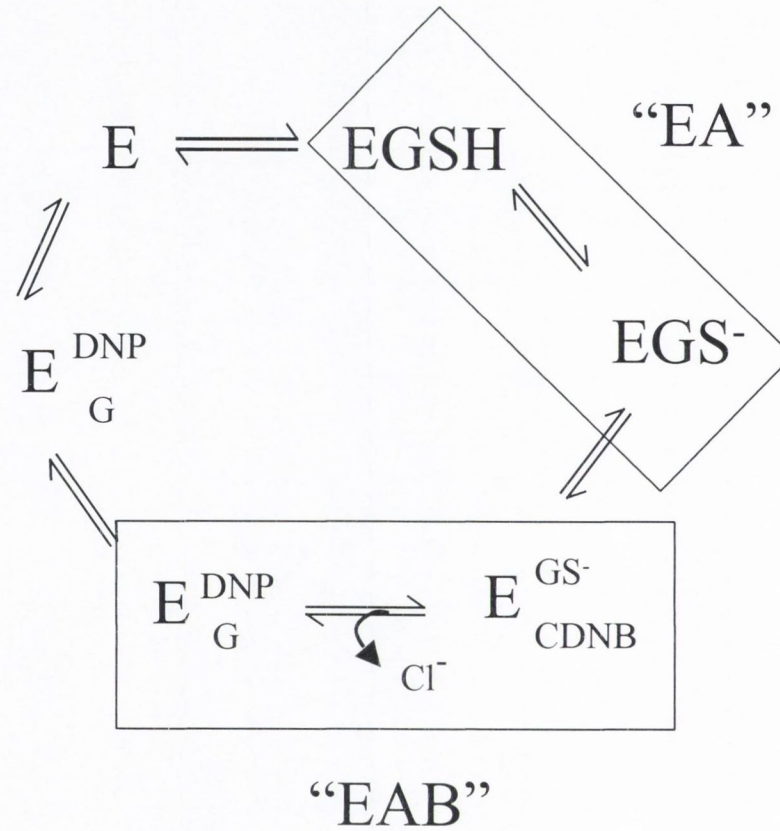
$$\frac{v_o}{E_t} = \frac{n [\text{A}] [\text{B}]}{\phi_A [\text{A}] + \phi_B [\text{B}] + \phi_{AB} [\text{A}] [\text{B}]}$$

Eq. 2

$$v_o = \frac{V_{\text{max}} [\text{A}] [\text{B}]}{K_B [\text{A}] + K_A [\text{B}] + [\text{A}] [\text{B}]}$$

Eq. 3

Scheme 2



**Scheme 3**

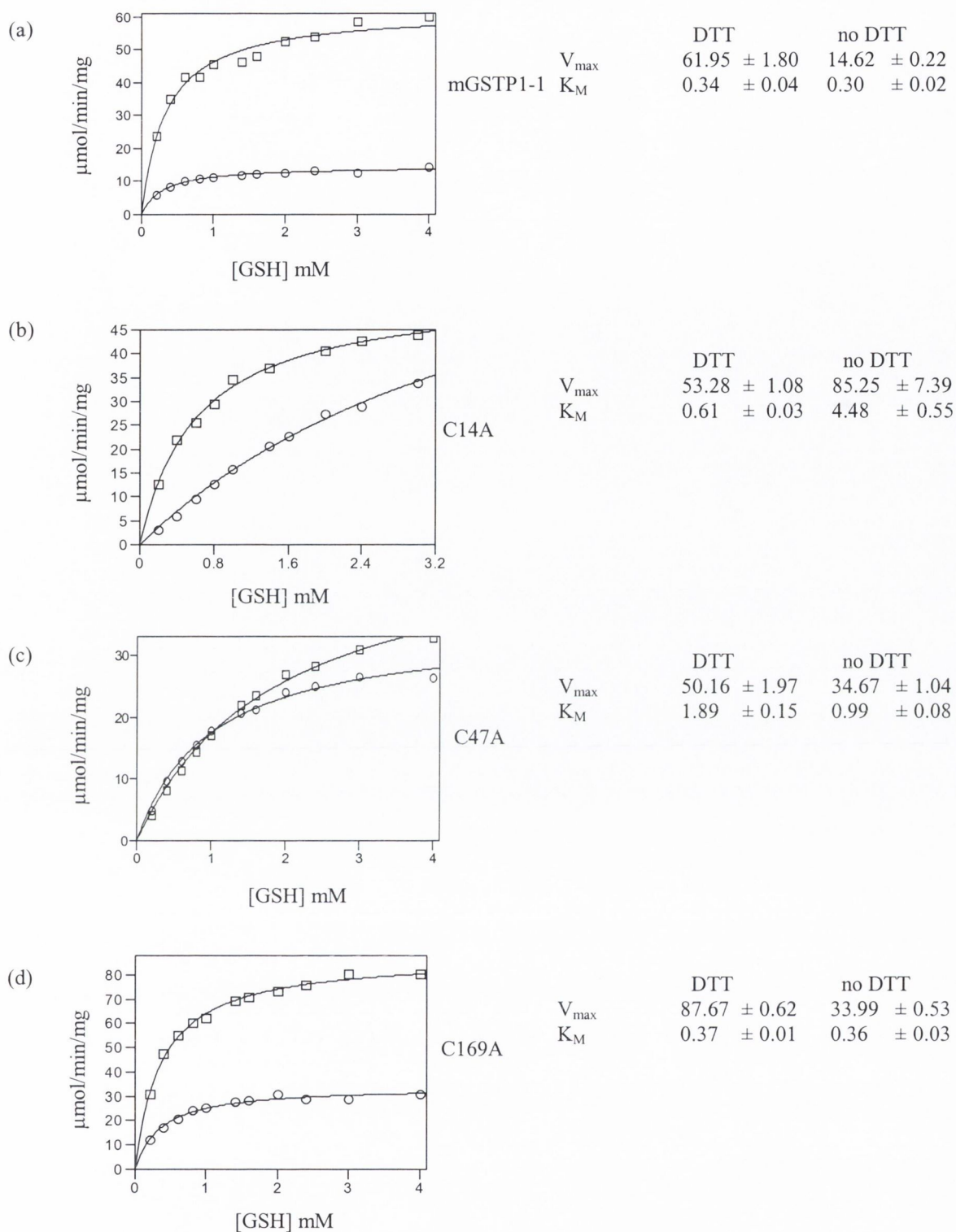
An ordered sequential representation for the mGSTP1 reaction. For the mutants C14A and C169A the deprotonation step, Denoted in box “EA” is assumed to be irreversible.



binding of CDNB the simple assumption that  $E.GS^-$  is formed irreversibly gives a mechanism that can now be treated as ping-pong (Scheme 2). The inclusion of the irreversible step results in a rate equation with no  $\phi_0$  term, therefore no  $K_{ia}K_B$  equivalent, and the prediction of families of parallel double reciprocal plots. As will be discussed more fully in Section 6, there is a major question regarding the mechanism by which these two mutants apparently extrude protons irreversibly.

As part of a possible explanation relies on structural data obtained by Maria Costa Entrade, Miquel Coll, Sinead Walsh and Tim Mantle (Trinity College Dublin unpublished work), this will be introduced here to facilitate later discussion. These workers have obtained the crystal structure of the C47A mutant complexed with *S*-(*p*-nitrobenzyl)-glutathione (Spn-GS) and this reveals two major features that are vitally linked to the current kinetic studies. Firstly, there is an asymmetry in the two crystallographic subunits (Fig. 4.6), which has implications for the observed cooperativity and, secondly, the difference has apparently frozen two ‘snapshots’ of the reaction mechanism, one with the crucial water molecule W0 bridging the Spn-GS and the Y7-hydroxyl group (a postulated precursor to expulsion of this water molecule) and a second ‘snapshot’, after expulsion of the W0 molecule and conjugation of the protein-bound Spn-GS (Fig 4.7)

All kinetics were performed on enzymes that had been stored at  $-20^{\circ}C$  in 2 mM DTT for less than 2 months and that were gel-filtered before used for kinetics. A comparison of mGSTP1-1 and various mutants stored at  $-20^{\circ}C$  in the absence of DTT (with and without DTT treatment) was carried out and some salient features are reported below. For the native enzyme, there was a four fold increase in the apparent  $V_{max}$  when the enzyme was treated with DTT (Fig. 4.5). The effects on C14A, C47A and C169A are also shown in Fig. 4.5. The changing of Cys-14 or Cys-47 to an

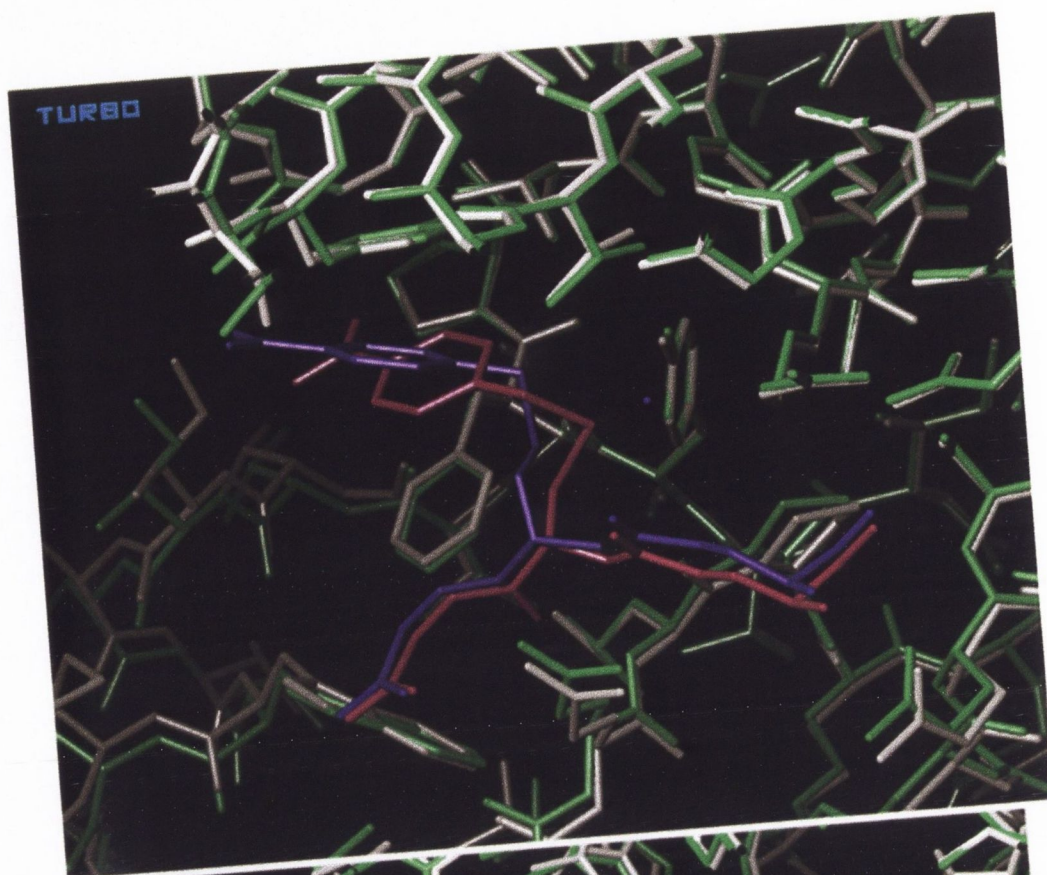


**Figure 4.5 Comparison of mGSTP1-1 and various mutants for activity after storage with and without DTT**

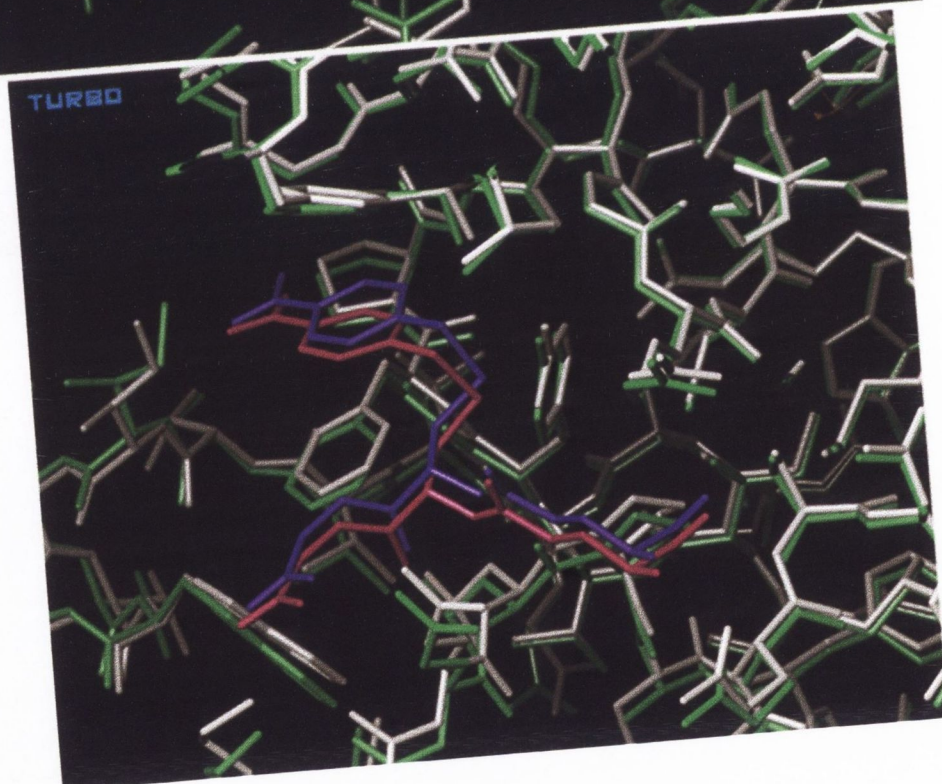
Kinetics using 1.5 mM CDNB and varying [GSH] after treatment with DTT (□) or in the absence of DTT (○) for (a) mGSTP1-1, (b) C14A, (c) C47A and (d) C169A. Values shown are apparent  $V_{\max}$  and apparent  $K_M$ . All the kinetics were determined in 0.1 M sodium phosphate pH 6.5 and at 30°C. The product was measured spectrophotometrically at 340 nm. The units for  $V_{\max}$  are  $\mu\text{mol}/\text{min}/\text{mg}$  and  $K_M$  are mM.



(1)



(2)

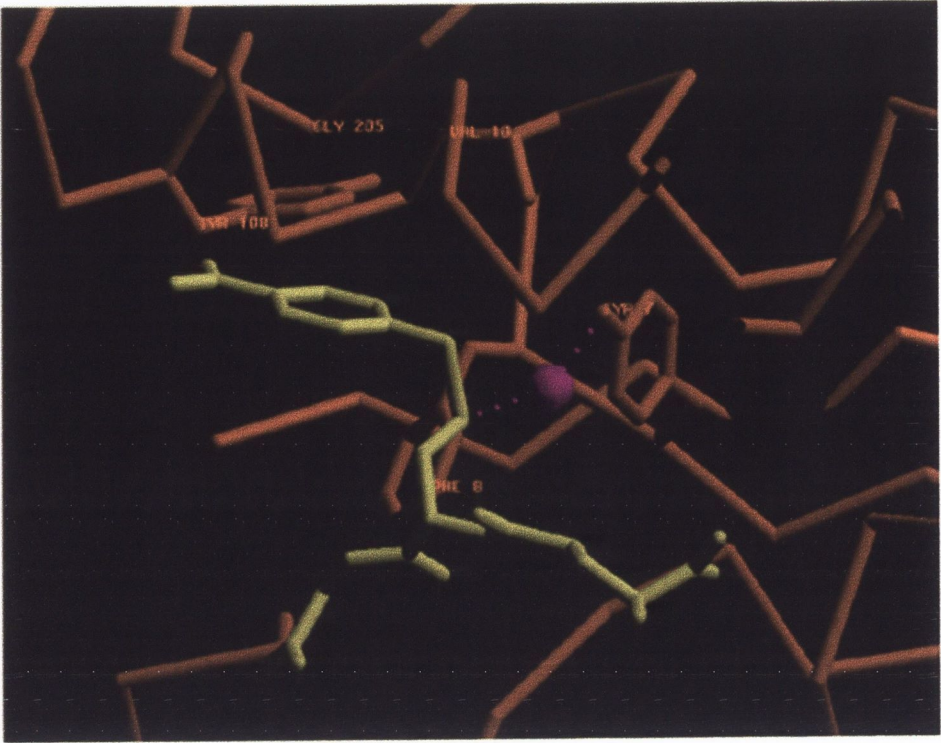




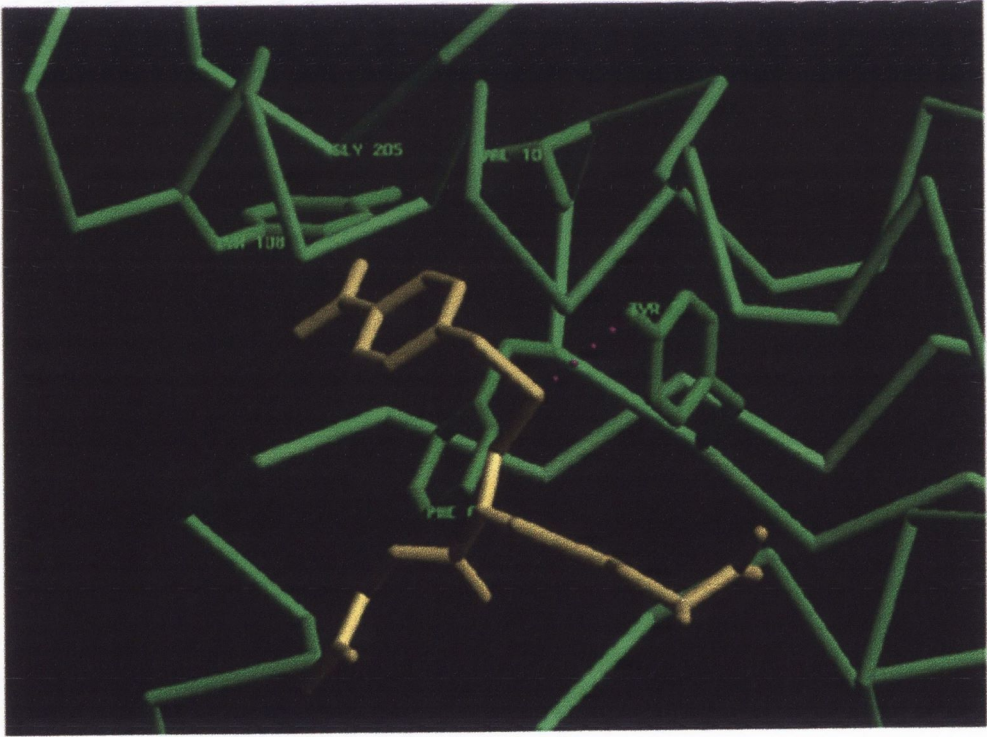
**Figure 4.6 Crystal structures of mGSTP1-1 and of the C47A mutant complexed with *S*-(*p*-nitrobenzyl)glutathione**

The structure of the active site for the two crystallographic subunits superimposed with the inhibitor *S*-(*p*-nitrobenzyl)glutathione. (1) The C47A mutant with the inhibitors *p*-nitrobenzyl moiety at an angle to each other. (2) The native mGSTP1-1 with the inhibitor in the two active sites aligned.

(1)



(2)



**Figure 4.7 Crystal structures of the two subunits of the C47A mutant complexed with *S*-(*p*-nitrobenzyl)glutathione**

The structure of the active site for the two subunits complexed with the inhibitor *S*-(*p*-nitrobenzyl)glutathione is shown. (1) The crystallographic subunit A with the inhibitor complexed and W0 still present. (2) The crystallographic subunit B with the inhibitor complexed and the W0 displaced, as is seen for native mGSTP1-1 with this inhibitor present.



alanine residue reduces the effect of DTT treatment on the apparent  $V_{\max}$  while the C169A mutant has a nearly three fold difference in  $V_{\max}$  and behaves in a similar manner to the native enzyme. There is a modest effect on the apparent  $K_m^{\text{GSH}}$  for the C47A mutant but no effect on the apparent  $K_m^{\text{GSH}}$  of the native enzyme or the C169A mutant. The C14A mutant reveals a marked increase in the apparent  $K_m^{\text{GSH}}$  when not stored in DTT. Basically, those forms with Cys-47 (native and C169A) appear to be oxidized on storage, with 60% (C169A) to 75% (native enzyme) of the enzyme appearing to be essentially inactive. However, the oxidation state is reversible on treatment with DTT.

#### **4.2 The binding of ligands to mGSTP1-1 and the various cysteine to alanine mutants using the quenching of protein fluorescence**

Quenching of the protein fluorescence upon addition of GSH to the enzyme was observed with both native enzyme and the various mutants (Table 4.3). This was carried out in 0.1 M sodium phosphate pH 6.5. The  $K_d$  values for the native and mutant mGSTP1-1 followed a similar pattern to the  $K_m^{\text{GSH}}$  values from Table 4.2. The fluorescence quenching of the inhibitor *S*-(*p*-nitrobenzyl)-glutathione was also measured. This compound was used because it binds both to the GSH (G-subsite) and the hydrophobic (H-subsite), as determined by crystallographic studies (Garcia-Sanez *et al.*, 1994). The  $K_d$  values for *S*-(*p*-nitrobenzyl)-glutathione binding to the native enzyme and the various mutants are shown in Table 4.3.

|       | GSH (mM)     | <i>S</i> -( <i>p</i> -nitrobenzyl)-glutathione (μM) |
|-------|--------------|-----------------------------------------------------|
| mPi   | 0.24 ± 0.02  | 1.47 ± 0.12                                         |
| C14A  | 0.32 ± 0.03  | 9.3 ± 0.71                                          |
| C47A  | 0.76 ± 0.08  | 5.26 ± 0.16                                         |
| C169A | 0.27 ± 0.025 | 9.67 ± 0.73                                         |
| C14*  | 0.63 ± 0.04  | 12.58 ± 0.27                                        |
| C47*  | 0.23 ± 0.006 | 7.49 ± 0.7                                          |
| C169* | 2.58 ± 0. 2  | 44.38 ± 0.5                                         |
| 3A    | 4.12 ± 0.38  | 34.53 ± 1.14                                        |

**Table 4.3**  $K_d$  values for GSH and Spn-GS binding to native mGSTP1-1 and to various mutants

The fluorescence of enzyme (100 μg in 0.1 M sodium phosphate pH 6.5) was measured using a Perkin-Elmer luminescence spectrometer. Successive amounts of ligand (either GSH or Spn-GS) were added and the change in fluorescence was recorded. The excitation wavelength was 295 nm and the emission wavelength was 345 nm. After allowing for dilution and correcting for the inner filter effect, the change in fluorescence was plotted against ligand concentration and fitted to a hyperbola (see Appendix II).

# **Chapter 5**



## 5.1 Introduction to glutathionylation

When the levels of reactive oxygen species (ROS) levels exceed cellular antioxidant capacity, a condition known as oxidative stress occurs. Unchecked, excessive ROS production can lead to the destruction of cellular components. Protein carbonylation is an irreversible oxidative modification of amino-acid side-chains into aldehyde or ketone groups, which can lead to protein aggregation, inactivation or degradation (Levine *et al.*, 2002). Under conditions of moderate oxidative stress, cysteine oxidation can lead to the formation of mixed disulfides between protein thiols and oxidized low molecular mass thiols, particularly glutathione (*S*-glutathionylation). This modification is reversible once the redox stress is removed and may serve an antioxidant role in protection of protein thiol groups against irreversible oxidation. ROS also play a role in cell signalling, with early studies implicating  $H_2O_2$  as an endogenous messenger (Sundaresan *et al.*, 1995). It has also been shown that glutathionylation may play a regulatory role analogous to other post-translational modifications such as protein phosphorylation. Lind *et al.* (1998) showed that glutathionylation was an oxidative modification of human glyceraldehyde-3-phosphate dehydrogenase. It has also been shown to effect signaling pathways once thought to be exclusively under the control of phosphorylation (Anselmo *et al.*, 2004).

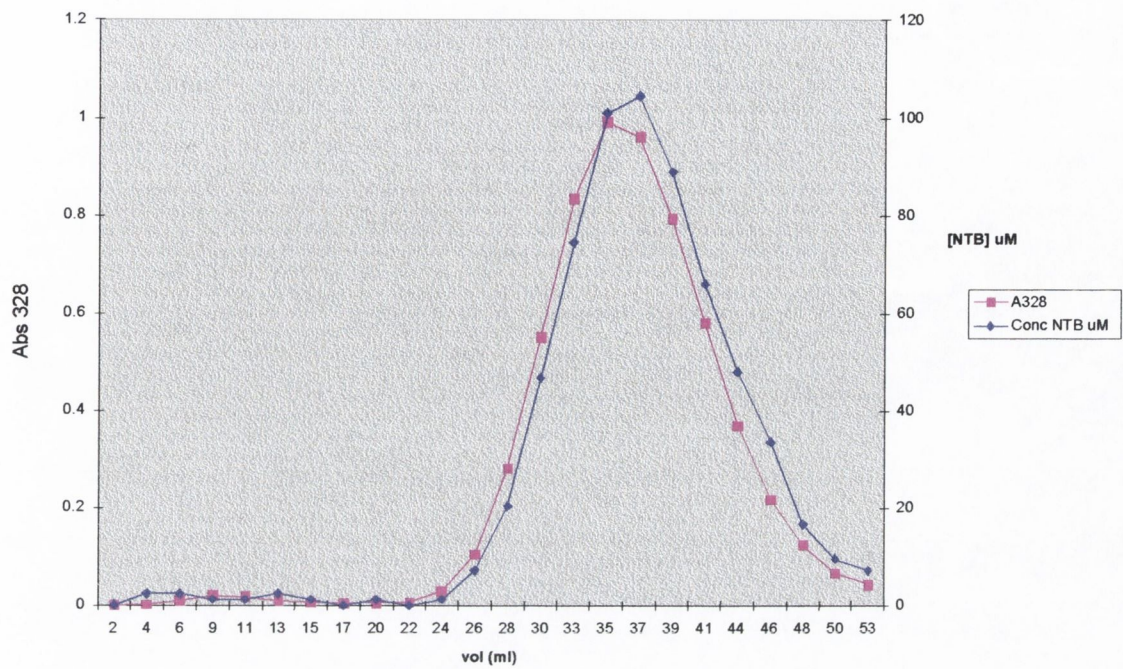
To study glutathionylation in more detail, a method is needed to allow the formation of stable protein mixed-disulfides with glutathione to allow glutathionylation and deglutathionylation to be studied. A method of glutathionylating specific proteins would also allow the effects of glutathionylation on the activity of the target protein to be studied. Such a system can also be used to screen for proteins

capable of removing glutathione from various donors to a range of potential acceptors.

For the formation of mixed disulfides with protein thiol groups, the protein can be subjected to an activation reaction, for example, with Ellman's reagent, yielding an activated mixed disulfide of the protein. In the second step, the activated mixed disulfide is reacted with the required thiol compound. The driving force of the reaction is the release of the stable NTB anion. The alternative is to make an activated mixed disulfide of the thiol and then react this with the protein. When these activated mixed disulfides are made with Ellman's reagent, the release of the NTB anion can be measured at 412 nm.

With the availability of the site-directed mutant forms of mGSTP1, engineered to have all possible arrangements of cysteine or alanine at positions 14, 47 and 169, and armed with the information from Section 3.5 on the reactivity of the thiol groups of cysteine-14, cysteine-47 and cysteine-169 with DTNB, it was decided to develop a reagent that would be useful to study the glutathionylation reactions of mGSTP1. Originally described by Mannervik (1970), the reaction of DTNB with glutathione yields GSSN where N is NTB. A quick and novel method to synthesize this reagent was developed using ion-exchange chromatography on DEAE-cellulose and this protocol was then adapted to synthesize  $G[^{35}\text{S}]SN$ . In theory, this reagent should be useful as a method for introducing radioactively labelled glutathione as a mixed disulfide with cysteine side chains and to conveniently monitor this process by measuring the release of NTB at 412 nm.

DEAE column GSSN



**Fig 5.1. Ion-exchange chromatography of GSSN on DEAE-cellulose**

GSSN is made by mixing GSH (4 mM) with DTNB (6 mM) and is then eluted off a DEAE cellulose column. The GSSN is monitored at  $A_{328}$  (■) while the  $A_{412}$  (◆) was monitored after addition of a 20 fold molar excess of GSH to the samples to release the NTB from GSSN.

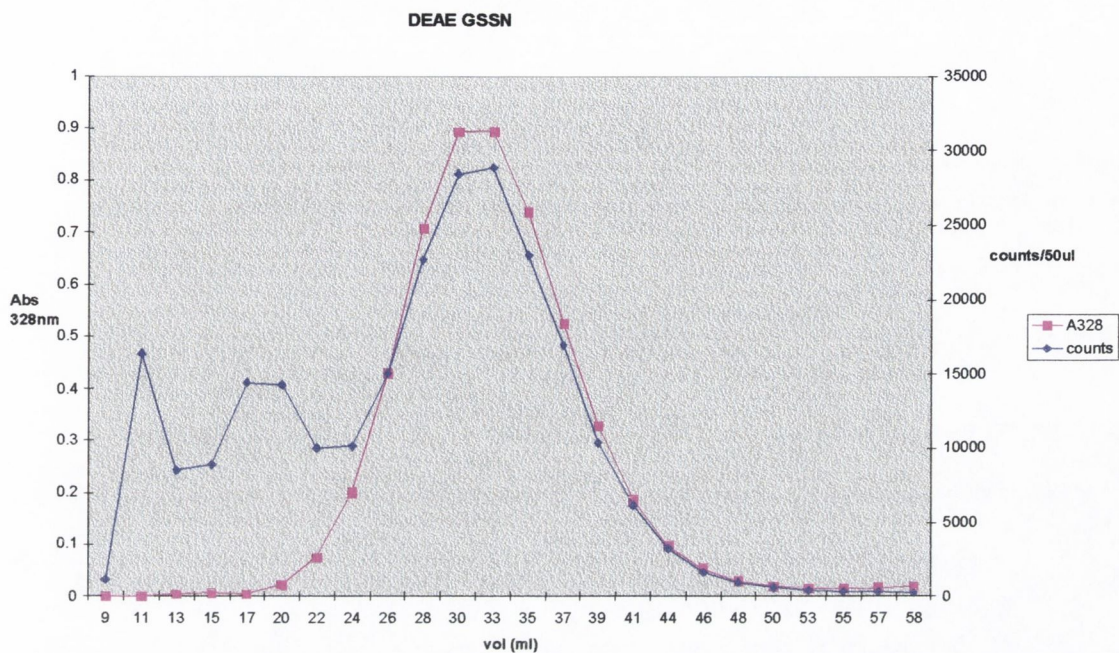


## 5.2 Purification of GSSN

GSSN mixed disulfide is made by mixing reduced glutathione (4 mM) with DTNB (6 mM). This reaction was carried out at pH 7.2 in 10 mM sodium phosphate buffer and goes to completion on mixing. When the reaction products are charged onto DEAE-cellulose equilibrated in 10 mM sodium phosphate pH 7.2, the yellow NTB is clearly retarded but does migrate slowly down the column. The fraction containing GSSN elutes cleanly before the NTB and the absorbance at 328 nm was measured as it eluted from the column, as shown in Fig. 5.1. The fractions were also mixed with GSH and the absorbance at 412 nm was measured to monitor the release of NTB anion (Fig. 5.1). The extinction coefficient for GSSN at 328 nm was found to be  $9530 \text{ M}^{-1}\text{cm}^{-1}$ . The stability of the GSSN was tested by keeping it stored at  $-20^{\circ}$  for one month. On thawing, the GSSN was mixed with glutathione and the release of NTB was measured at 412 nm. The result was identical to that obtained for the freshly prepared material.

## 5.3 Reaction of GSSN with the native and various cysteine to alanine mutants of mGSTP1

A sample (20  $\mu\text{M}$ ) of native mGSTP1 was incubated with 100  $\mu\text{M}$  GSSN in a final volume of 1 ml sodium phosphate pH 7.2. The reaction went to completion within 10 min and liberated a little over 20  $\mu\text{M}$  NTB per monomer. This suggests the GSSN has reacted with cysteine-47 which was shown previously to be the uniquely reactive thiol at neutral pH (see Section 3.5), with a small amount of NTB release due to the less reactive cysteine-169. This was confirmed when a sample of the mutant



**Fig 5.2. Ion-exchange chromatography of G[<sup>35</sup>S]SN on DEAE-cellulose**

Glutathione (4 mM; 1 µCi) was mixed with 6 mM DTNB in a final volume of 1 ml and then charged on DEAE-cellulose column as described in Section 6.4. The GSSN was monitored at A<sub>328</sub> (■) and radioactivity (♦) by scintillation counting.

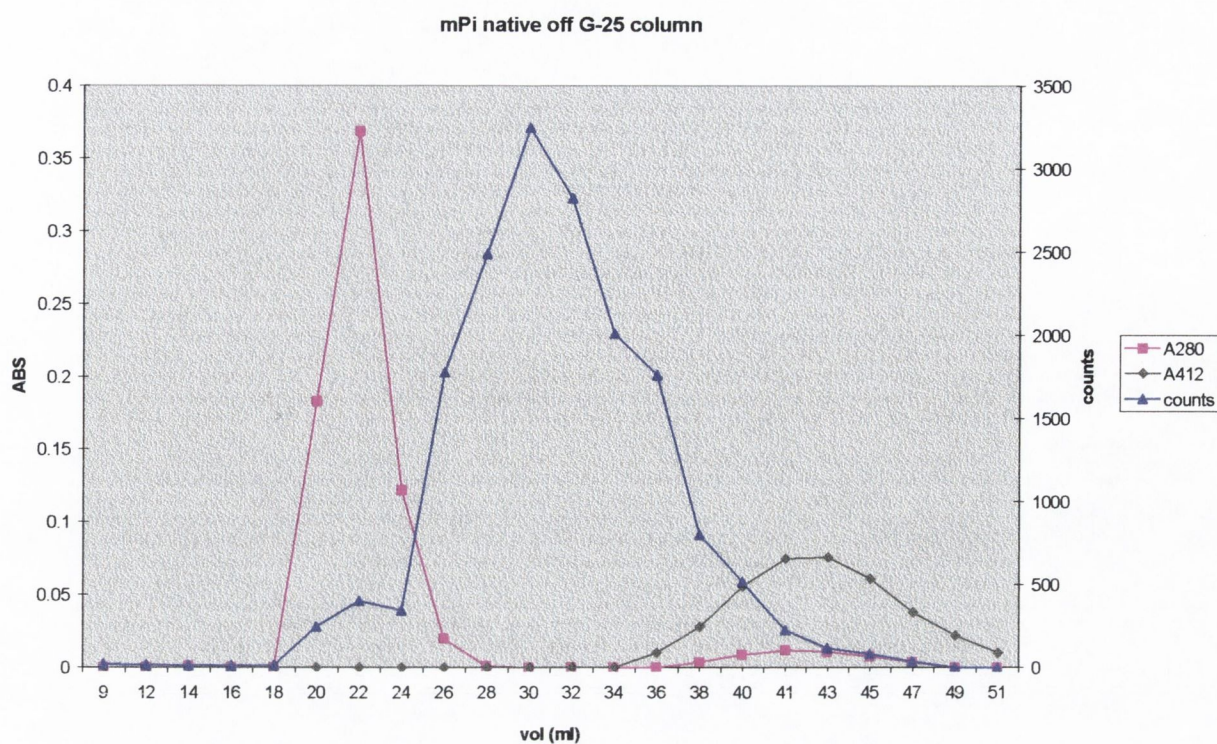
C47A enzyme (20  $\mu$ M) was incubated with 100  $\mu$ M GSSN. Almost no release of NTB occurs. This was repeated for C14A and C169A, which showed similar results to those obtained for the native mGSTP1. This suggested that the glutathione from GSSN was forming a disulfide with the protein thiol (Cys-47) and releasing NTB.

#### **5.4 Making GSSN with [ $^{35}$ S] glutathione to measure the fraction of glutathione bound to mGSTP1**

The release of NTB suggests that the thiol on the protein forms a mixed disulfide with glutathione, but this is difficult to quantify directly. GSSN was prepared as before but [ $^{35}$ S]-labelled glutathione was added to allow preparation of G[ $^{35}$ S]SN. The absorbance of fractions eluted from the DEAE column was measured at 328 nm and the radioactivity was also measured (Fig. 5.2). The absorbance peak at 328 nm corresponded to the peak in radioactivity, with some smaller peaks of radioactivity probably being due to GSSG or unreacted GSH.

The purified GSSN (100 nmoles) was reacted with mGSTP1 (50 nmol) as before in a final volume of 1 ml and the NTB released was measured. Approximately 1.2 mol of NTB released per mole of monomer. This was then gel filtered on a Sephadex G-25 column to separate the protein from the unbound GSH and unreacted GSSN. The absorbance of the fractions was measured at 280 nm and also counted in the scintillation counter (Fig. 5.3). There was a small amount of radioactivity that corresponded to the protein peak but most of the radioactivity followed after the protein, indicating that the glutathione was not bound (Table 5.1). The main peak of radioactivity came after the protein but before the NTB. This was most likely to be





**Fig 5.3 Gel filtration of mGSTP1 after incubation with G[<sup>35</sup>S]SN.**

mGSTP1 (50 nmol) was incubated with G [<sup>35</sup>S]SN (100 nmol) which had a specific activity of 11 dpm per pmol, in a final volume of 1 ml, for 10 min at room temperature and then passed down a Sephadex G-25 column. The protein was monitored at A<sub>280</sub> (■), the released NTB was monitored at A<sub>412</sub> (◆) and radioactivity was measured (▲) by taking 50 µl samples for scintillation counting. For the peak fraction of protein, the 50 µl sample measured contained 750 pmol of monomer, which incorporated 402 dpm (750 pmol of bound glutathione should give 8340 dpm), which equates to 0.048 mol of glutathione bound per mole of protein monomer.

| Protein         | Molar fraction of GS per mole of monomer |
|-----------------|------------------------------------------|
|                 |                                          |
| GST P1-1 native | 0.048                                    |
| C169A           | 0.167                                    |
| C47*            | 0.466                                    |
| C14A            | 0.535                                    |

**Table 5.1      Incorporation of glutathione measured using [<sup>35</sup>S] GS<sup>-</sup> bound to protein after it was eluted from a G-25 column**

Protein was mixed with G[<sup>35</sup>S]SN at pH 7.2 and filtered on a Sephadex G-25 column. The fraction of moles of glutathione per mole of protein monomer was calculated from the amount of radioactive glutathione bound to protein. This is a representative data set from two experiments.

GSSG, as, when an excess of GSH was reacted with  $G[^{35}\text{S}]\text{SN}$  and filtered on a Sephadex G-25 column the radioactivity emerges at the same position (Fig. 5.4).

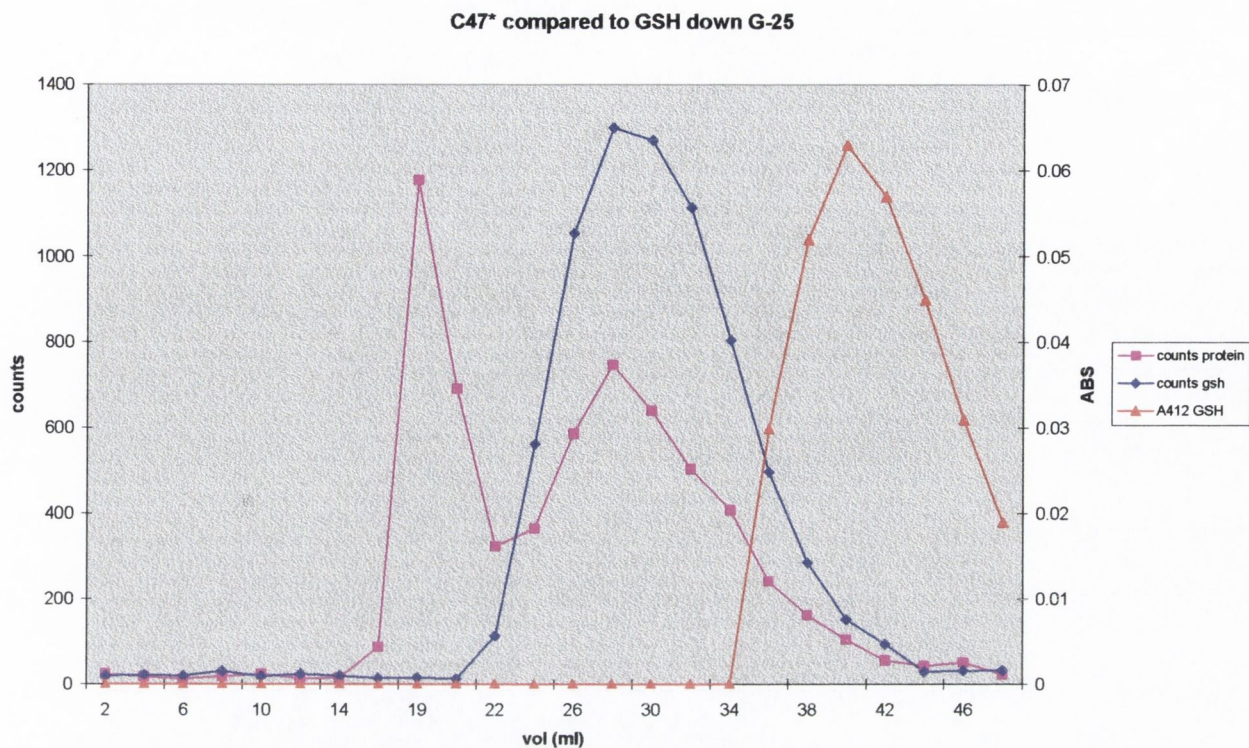
This experiment was repeated with biliverdin reductase A (BVRA) to see if this was just an effect on GSTs. BVRA was mixed with  $G[^{35}\text{S}]\text{SN}$  and release of NTB was measured as before. This mixture was then passed down a Sephadex G-25 column and the amount of bound glutathione was measured as above, with only 0.03 mol of glutathione being bound per mole of BVRA. The small amount of glutathione bound showed it was not an effect specific to GSTs.

To simplify the experiment, the C47\* mutant, which has only the one thiol per monomer was used. This was treated with  $G[^{35}\text{S}]\text{SN}$  as above and filtered on a Sephadex G-25 column and was found to have approximately half a mol of glutathione per mol of monomer (0.46). This suggests that the more thiols present, the more likely they are to cross react and displace the bound glutathione as GSSG. The final mol fraction of approximately half glutathione per mol of monomer is initially at odds with the greater release of NTB per mol of monomer. A mechanism to reconcile these observations is shown in Scheme 5.1.

A sample of C169A mutant was reacted with  $G[^{35}\text{S}]\text{SN}$  as above and gel filtered on Sephadex G-25. The absorbance at 280 nm and radioactivity were measured for each fraction (Fig. 5.5). A similar result was obtained to that found with the native mGSTP1 but with slightly more glutathione bound to the protein (Table 5.1). Therefore, removing the cysteine-169 improved, but did not have a major influence on, the molar fraction of glutathione per monomer.

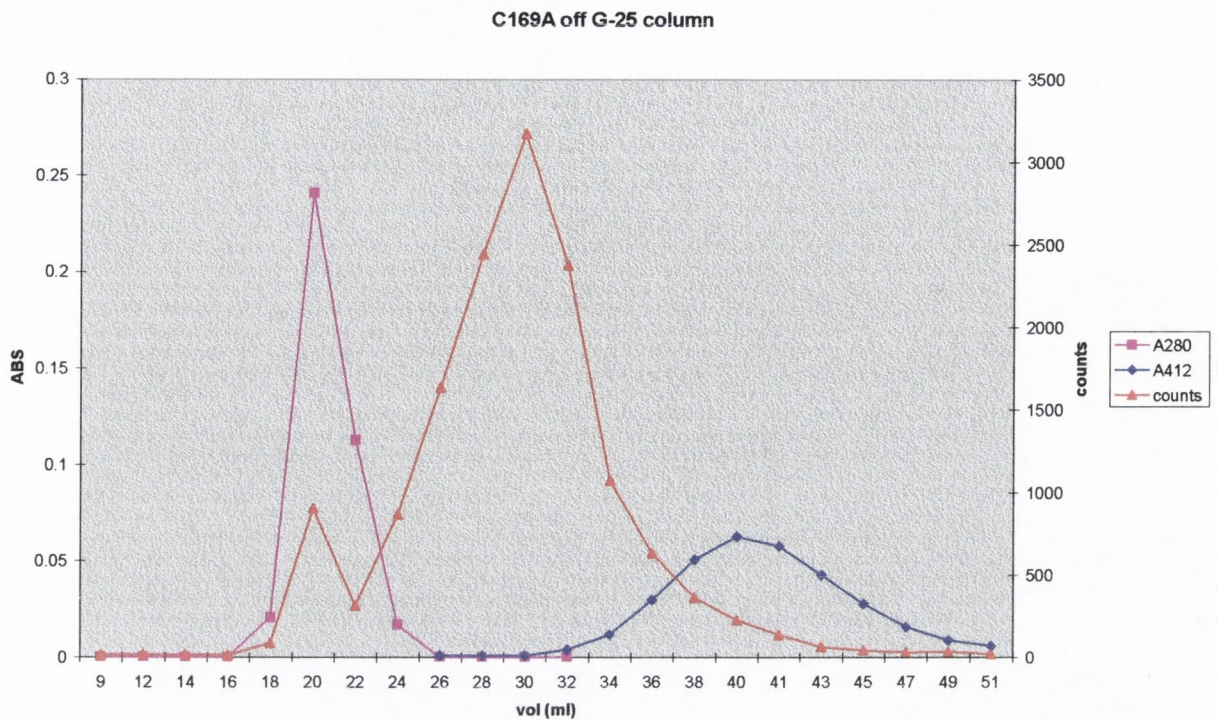
Cysteine-14 is buried in the active site and does not appear to contribute to the reaction with GSSN of the wild-type mGSTP1, as the C47A mutant does not release





**Fig 5.4 Gel filtration comparison of C47\* and GSH both incubated with GS[<sup>35</sup>S]N**

The C47\* single thiol mutant was incubated with G[<sup>35</sup>S]SN for 10 min at room temperature before being gel-filtered on a Sephadex G-25 column. The radioactivity was monitored by taking 50 µl samples for scintillation counting (■). GSH was mixed with G[<sup>35</sup>S]SN before being gel-filtered on the same Sephadex G-25 column and the radioactivity was monitored by scintillation counting (◆) and NTB released was monitored at A<sub>412</sub> (▲)



**Fig 5.5 Gel filtration chromatography of C169A after incubation with G[<sup>35</sup>S]SN**

C169A mutant (40 nmol) was incubated with G[<sup>35</sup>S]SN (100 nmol) that had a specific activity of 11 dpm per pmol, in a final volume of 1 ml for 10 min at room temperature and was gel-filtered on a Sephadex G-25 column. The protein was monitored at A<sub>280</sub> (■), and the released NTB was monitored at A<sub>412</sub> (◆). The radioactivity was monitored with 50 µl samples by scintillation counting (▲). For the peak fraction of protein the 50 µl sample measured contained 490 pmol of monomer which incorporated 903 dpm. As 490 pmol of bound glutathione should give 5448 dpm, 490 pmol equates to 0.16 mol of glutathione bound per mole of protein monomer.



NTB from GSSN. However, when a sample of C14A was reacted with  $G[S^{35}]SN$  and gel-filtered on a Sephadex G-25 column, it was found to have a similar molar fraction of glutathione bound per monomer as the C47\* mutant (Table 5.1). This unexpected result is discussed more fully in Section 6.

## 5.5 Testing reactivity with added cationic detergent

The chemically active species of a protein thiol is the ionized form of the sulfhydryl, the thiolate anion. Protein thiol pKa values vary over a wide range depending on their environment, with most having values in the region of 8.5. The low pKa values of some protein thiols are believed to result from the environmental effects of proximal cationic groups of lysine, arginine and histidine. The positively charged groups lower the pKa of the thiol group by stabilizing the thiolate anion as an ion pair. Labelling in the presence of additives that increase positive charge density next to the protein sulfhydryl groups should increase the reactivity. One such reagent is the cationic detergent hexadecyltrimethylammonium bromide, also known as cetyltrimethylammonium bromide (CTABr), which binds to proteins enhancing the local positive charge density and has been reported to decrease sulfhydryl pKa values by one or two pH units (Gitler *et al.*, 1995). Binding of the detergent may also result in conformational changes that may expose buried thiol groups. The reaction of the single thiol mutants of mGSTP1 with DTNB in the presence of 2 mM CTABr was followed by measuring the absorbance at 412 nm and the results are shown in Table 5.2. The use of the cationic detergent increased the reactivity of cysteine-14 and cysteine-169, as seen by the large increase in NTB release. There is a decrease in



| Protein | Molar fraction of NTB<br>release per mole of monomer<br>in the absence of CTABr | Molar fraction NTB release<br>per mole of monomer in<br>presence of CTABr |
|---------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------|
| C14*    | 0.005                                                                           | 0.592                                                                     |
| C47*    | 0 .728                                                                          | 0.505                                                                     |
| C169*   | 0 .057                                                                          | 0.557                                                                     |

**Table 5.2 Release of NTB in the presence and absence of the cationic detergent CTABr for protein mixed with DTNB**

The single cysteine mutants of mGSTP1-1 were incubated at pH 7 in 0.1 M sodium phosphate (with and without 2 mM CTABr) with DTNB. The NTB released was measured spectrophotometrically at 412 nm. This is a representative data set from two experiments.

NTB release for the C47\* mutant, which may be due to formation of protein-protein disulfides before DTNB can attack one of the thiols.

The experiments were repeated as in Section 5.4 but with the addition of CTABr (2 mM) for the single thiol mutants of mGSTP1 and the native protein. The NTB release and molar fraction of glutathione per mole of monomer were measured (Table 5.3). There was a dramatic increase in the amount of glutathione that bound to the protein. The native mGSTP1 went from 0.048 mol of glutathione bound per mole of monomer up to 1.053, which may be accounted for in part by the increase in activity of cysteine-14 and cysteine-169. The only mutant to be adversely effected by the detergent was C47\* which only binds 0.1785 mol of glutathione per mole of monomer.

| Protein | Molar fraction of glutathione per mole of monomer | Molar fraction of NTB released per mole of monomer |
|---------|---------------------------------------------------|----------------------------------------------------|
| mPi     | 1.05                                              | 1.65                                               |
| C14*    | 0.35                                              | 0.69                                               |
| C47*    | 0.18                                              | 0.77                                               |
| C169*   | 0.33                                              | 0.69                                               |

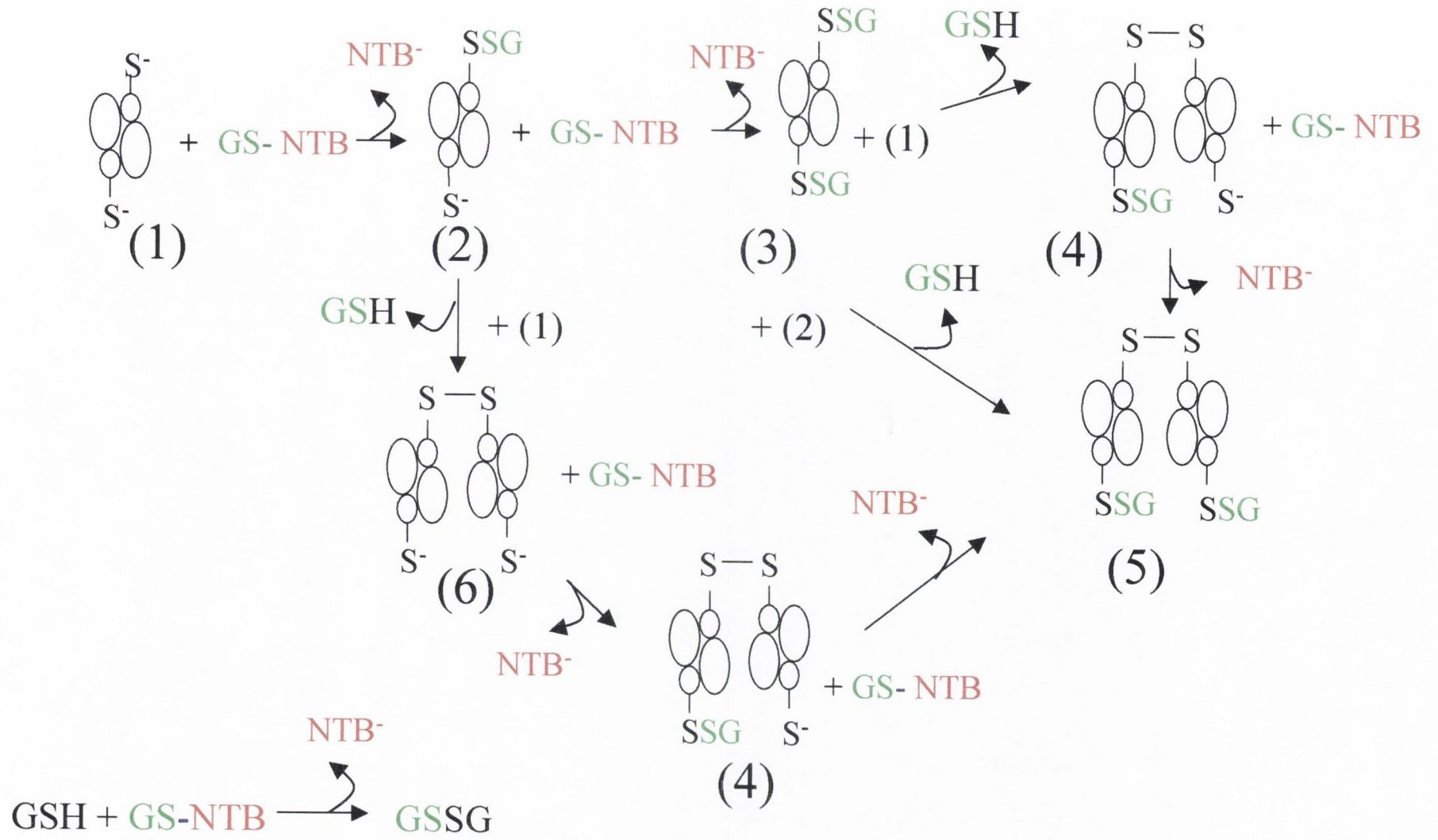
**Table 5.3 mGSTP1 and single thiol mutants mixed with G[<sup>35</sup>S]SN in the presence of CTABr**

mGSTP1-1 wild type and the single-cysteine mutants were incubated at pH 7 in 0.1 M sodium phosphate and 2mM CTAB with GSSN. The molar fraction of glutathione per mole of monomer of enzyme was measured by scintillation counting and the NTB released was measured spectrophotometrically at 412 nm. This is a representative data set from two experiments.



### **Scheme 5.1 Reactions of GSSN (GS-NTB) with C47\*.**

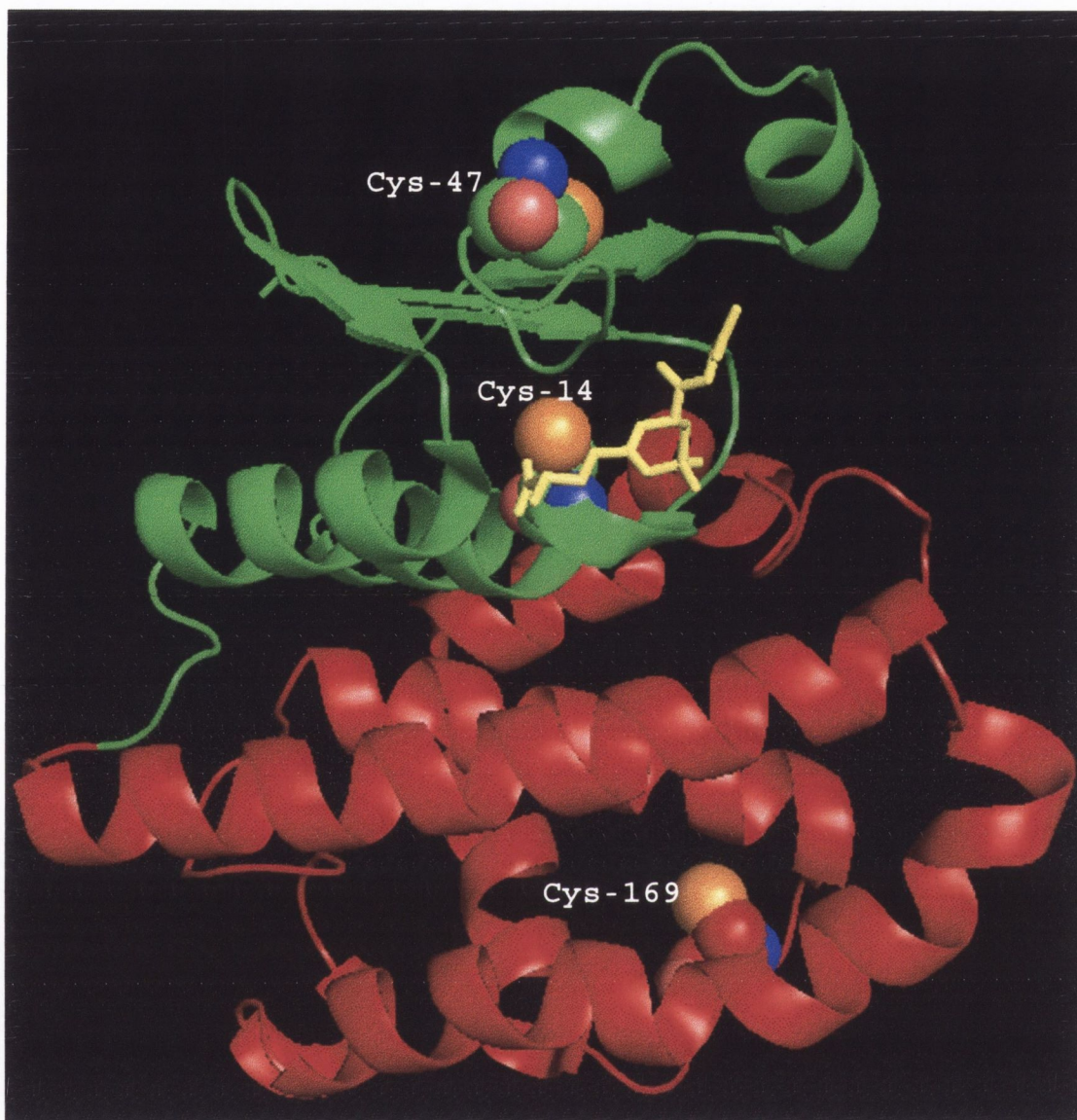
A Scheme to reconcile the release of approximately 1 mol of NTB per mol of subunit of C47\* but only a half mol of glutathione bound to the protein. The initial reaction of the C47\* mutant which has one reactive thiol per monomer with GSSN (in this case depicted as GS-NTB for graphical purposes) gives release of NTB with the glutathione forming a disulfide with the cysteine thiol (2). This can then further react with another GSSN to form a protein with both thiols glutathionylated (3). But at the same time these glutathionylated thiols appear to be more reactive to the free thiols on other dimers in solution which have not yet reacted with GSSN and these form disulfides between dimers with the release of glutathione. The free glutathione can react with more GSSN to form GSSG and release more NTB thereby increasing the apparent value for glutathionylation worked out from the absorbance change at 412 nm. This process continues till all free thiols are reacted.



# Chapter 6



mGSTP1-1 is a dimeric enzyme composed of identical subunits, each of which contains one binding site for glutathione (G-site) and one hydrophobic binding site for the co-substrate (H-site), which, in the present study, was 1-chloro-2,4-dinitrobenzene (CDNB). Steady-state kinetics of the native enzyme are strictly hyperbolic for both these substrates at high concentrations of CDNB. However, all of the cysteine-to-alanine mutants exhibit some degree of cooperativity and even the native enzyme exhibits Hill coefficients greater than one at low concentrations of CDNB. Thus, it appears that the native structure may have evolved to suppress an intrinsic cooperativity. The crystal structures of all of the main classes of GSTs have been solved and provide useful data for elucidating the catalytic mechanism. From the structures, it is evident that all of these proteins have a similar fold. Fig. 6.1 shows one subunit of mGSTP1-1 in complex with glutathione, which is folded into two domains. Domain I (residues 1-74), which is coloured green in the photograph, consists of a central four-stranded  $\beta$ -sheet flanked on one side by an  $\alpha$ -helix and on the other side by an irregular helix structure that faces the solvent. The folding pattern resembles that of a thioredoxin fold. Domain II (residues 81-207), which is coloured red in the photograph, consists of five  $\alpha$ -helices. The mGSTP1-1 enzyme has three cysteines per subunit, at positions 14, 47 and 169. In all other mammalian GSTP1-1 isoenzymes where the sequence is known, there is also a cysteine at position 101 (Fig. 6.2). The crystal structure shows that Cys-47 is at a distance of 12 Å from the active-site pocket (bound glutathione sulfonate is coloured yellow) and is located on helix  $3_{10}B$  at the C-terminus of helix  $\alpha B$  ( $\alpha 2$  loop, residues 35-51). As can be seen in Fig. 6.1, Cys-14 is positioned behind the active site and Cys-169 is at the far end of the subunit on the outside of Domain II and away from the active site. However, it is only 31.8 Å from the active site of the other subunit of the dimer. This aspect is highlighted



**Fig 6.1 Ribbon structure of mGSTP1-1 monomer**

The ribbon structure of mGSTP1-1 monomer complexed with glutathione sulfonic acid. Domain I is in green and Domain II is in red. The cysteines are represented by spheres, red for oxygen, blue for nitrogen and orange for sulfur.



|                      |                                                                                   |
|----------------------|-----------------------------------------------------------------------------------|
| Mouse                | -PPYTIVYFPVRGR <b>C</b> EAMRMLLADQGQSWKEEVVTI-DTWMQGLLKPT <b>C</b> L              |
| Rat                  | -PPYTIVYFPVRGR <b>C</b> EATRMMLADQGQSWKEEVVTI-DVWLQGSILKST <b>C</b> L             |
| Hamster              | -PPYTIVYFPVRGR <b>C</b> EAMRILLADQGQSWKEEVVTV-ETWRKGSILKST <b>C</b> L             |
| Oranutan             | MPPYTIVYFPVRGR <b>C</b> AALRMLLADQGQSWKEEVVSM-ETWQEGSLKAS <b>C</b> L              |
| Macaque              | -PPYTIVYFPVRGR <b>C</b> AALRMLLADQGQSWKEEVVSM-ETWQEGSLKAS <b>C</b> L              |
| Human                | MPPYTIVYFPVRGR <b>C</b> AALRMLLADQGQSWKEEVVTV-ETWQEGSLKAS <b>C</b> L              |
| Bovine               | -PPYTIVYFPVQGR <b>C</b> EAMRMLLADQGQSWKEEVVAM-QSWLQGPLKAS <b>C</b> L              |
| Goat                 | -ASYTIVYFPVQGR <b>C</b> EAMRMLLADQGQSWKEEVVAM-QSWLQGPLKAS <b>C</b> L              |
| Pig                  | -PPYTIVYFPVRGR <b>C</b> EAMRMLLADQGQSWKEEVVSM-ETWP--PLKPS <b>C</b> L              |
| Toad                 | -PEYTIYFNARGR <b>C</b> EAMRMLMADQGAQWKEEVVTS-DDWQKGDILKAAV                        |
| Zebrafish            | MAPYTLTYFAVKGR <b>C</b> GALKIMLADKDQQLKENLVTF-EEWMKGDILKAT <b>C</b> V             |
| Salmon               | MGPYTLTYFVGRGR <b>C</b> GALRIMMADQGGQEWKEILMDF-ADWTKGDLKAS <b>C</b> V             |
| Mussel               | --MPTLVYFPVRGRAEAMRTLLTDQNTITYEEVNAGPRENWE-NWKPKMQ                                |
| Clam                 | MSKYQLFYFPVRGRGQAIRYLFVDNGLDYEEIDVGG--NWAT-EYKSKMP                                |
| Wuchereria bancrofti | -MSYKLTYPFIRGLAEPFIRLVLDQGIKFTDDRINA-SDWP--SMKSHFH<br>: * * : * . : : * * . : * * |

|                      |                                                                                                                   |
|----------------------|-------------------------------------------------------------------------------------------------------------------|
| Mouse                | YGQLP <b>K</b> FEDGD-LTLYQSNAILRHLGRSLGLYGKNQREAAQMDMVNDGVE                                                       |
| Rat                  | YGQLP <b>K</b> FEDGD-LTLYQSNAILRHLGRSLGLYGKDKQEAALVDMVNDGVE                                                       |
| Hamster              | YGQLP <b>K</b> FEDGD-LTLYQSNAILRHLGRSLGLYGKDKQEAALVDMVNDGVE                                                       |
| Oranutan             | YGQLP <b>K</b> FQDGD-LTLYQSNITILRHLGRTLGLYGKDKQEAALVDMVNDGVE                                                      |
| Macaque              | YGQLP <b>K</b> FQDGD-LTLYQSNITILRHLGRTLGLYGKDKQEAALVDMVNDGVE                                                      |
| Human                | YGQLP <b>K</b> FQDGD-LTLYQSNITILRHLGRTLGLYGKDKQEAALVDMVNDGVE                                                      |
| Bovine               | YGQLP <b>K</b> FQDGD-LTLYQSNAILRHLGRTLGLYGKDKQEAALVDMVNDGVE                                                       |
| Goat                 | YGQLP <b>K</b> FQDGD-LTLYQSNAILRHLGRTLGLYGKDKQEAALVDMVNDGVE                                                       |
| Pig                  | FRQLP <b>K</b> FQDGD-LTLYQSNAILRHLGRSFLYGKDKQEAALVDMVNDGVE                                                        |
| Toad                 | YGQLPGFKDGD-FTLYQSNAMLRLLARNHDLYGKNPREASLIDMVNDGVE                                                                |
| Zebrafish            | FGQLP <b>K</b> FEDGD-LVLFQSNAMLRHLGRKHAAYGKNDSEASLIDMVNDGVE                                                       |
| Salmon               | FGQLP <b>K</b> FEDGG-LVLYQSNAILRHLGRNHDAYGKDGKEAARIDMMCDGVE                                                       |
| Mussel               | FGQ <b>C</b> P <b>C</b> YTDGDFQVLVQSNAILAYLGRKYDLYGSDIKQASILDMMNSAVE                                              |
| Clam                 | FGQVPVVKDGD-LDVAQSNAIMRYLGRKHDMYPTDPVEALKVDMILDHSE                                                                |
| Wuchereria bancrofti | FGQLP <b>C</b> LYDGD-HQIVQSGAILRHLARKHNLNGGNELETTHIDMF <b>C</b> EGIR<br>: * * * . : * * : : * * . : : : * * . . . |

|                      |                                                                                                 |
|----------------------|-------------------------------------------------------------------------------------------------|
| Mouse                | DLRGKYVTLIYTNENYENGKNDYVKA-LPGHLKPFETLLSQNQGGKAFIVGD                                            |
| Rat                  | DLR <b>C</b> KYGTLIYTNENYENGKDDYVKA-LPGHLKPFETLLSQNQGGKAFIVGN                                   |
| Hamster              | DLR <b>C</b> KYITLIYTKYEEGKDDYVKA-LPGHLKPFETLLSQNQGGKAFIVGD                                     |
| Oranutan             | DLR <b>C</b> KYLSLIYTNYEAGKDDYVKA-LPGQLKPFETLLSQNQGGKTFIVGD                                     |
| Macaque              | DLR <b>C</b> KYLSLIYTNYEAGKDDYVKA-LPGQLKPFETLLSQNQGGKTFIVGD                                     |
| Human                | DLR <b>C</b> KYISLIYTNYEAGKDDYVKA-LPGQLKPFETLLSQNQGGKTFIVGD                                     |
| Bovine               | DLR <b>C</b> KYVSLIYTNYEAGKEDYVKA-LPQHLKPFETLLSQNKGGQAFIVGD                                     |
| Goat                 | DLR <b>C</b> KYVSLIYTNYQAGKEDYVKA-LPQHLKPFETLLSQNKGGQAFIVGD                                     |
| Pig                  | DLR <b>C</b> KYATLIYTNYEAGKEKYVKE-LPEHLKPFETLLSQNQGGQAFVVG                                      |
| Toad                 | DLRLKYLKMIYQNYENGKDDYVKA-LPTNLGHFERLLASNNEGKGFVVG                                               |
| Zebrafish            | DLRLKYIKLIYQYETGKEAFIKD-LPNHL <b>C</b> FENVLAKNKTG--FLVGD                                       |
| Salmon               | DLRLKYGKMIYQYETGKDQYIKD-LPNHLDKFEAVMAKNKTG--FLVGD                                               |
| Mussel               | DIRGAYVRMIYQNYEAGKEPFIFKE-LPEKLQPFENLLKPTKG--YILGE                                              |
| Clam                 | DIRGPYVRMIYQNYEAGKDDFIKS-LPEKFQYLENLLKNWGT--DFISS                                               |
| Wuchereria bancrofti | DLHTKYAKMIYQAYDTEKDSYIKDILPVELAKFEKLLATRDDGKNFILGE<br>* : : * : * * : * : : * * : : * : : . . . |



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Mouse      QISFADYNLLDLLLIHQVLAPGCLDNFPLLSAYVARLSARPKIKAFLSSP
Rat        QISFADYNLLDLLLVHQVLAPGCLDNFPLLSAYVARLSARPKIKAFLSSP
Hamster    QISFVDYNLLDLLLIHQVLAPGCLDNFPLLSAYVARLSARPKIKAFLSSP
Oranutan   QISFADYNLLDLLLIHEVLAPGCLDAFPLLSAYVARLSARPKLKAFLASP
Macaque    QISFADYNLLDLLLIHEVLAPGCLDAFPLLSAYVARLSARPKLKAFLASP
Human      QISFADYNLLDLLLIHEVLAPGCLDAFPLLSAYVGRLSARPKLKAFLASP
Bovine     QISFADYNLLDLLRIHQVLAPSCLDSFPLLSAYVARLNSRPKLKAFLASP
Goat       QISFADYNLLDLLRIHQVLAPSCLDSFPLLSAYVARLNSRPKLKAFLASP
Pig        QISFADYNLLDLLRIHQVLNPSCLDAFPLLSAYVARLSARPKIKAFLASP
Toad       HISFADYNLVDLLHNLVLAPDCLSGFPLLCAYVKRISSRPKLEAYLSSD
Zebrafish  QISFADYNLFDLLLNLKVLSPSCLDSFPSLKSFVDKISARPKVKALLECE
Salmon     KPSFADYSLFEVLLNHLVLCSCLDTSPLSKSFVEKMSARPKIKAFLGSD
Mussel     KISWVDYNLFDLLDILNLSPGCLDAFPVKAIFYERVILARPGVQKRRQTD
Clam       KISFADYSLFDDLLDVLILAPGCIDDFPTVKAYYDRIASRPALQKFRESE
Wuchereria KISYVDFVLFEELDIHQILDPHCLDKFPLLKAYHQMEDRPGLKEYCKQR
bancrofti  : *:.*: *.: *      :*. *. . * : :: :: ** ::

Mouse      EHVNRPINGNGKQ-----
Rat        DHLNRPINGNGKQ-----
Hamster    DHVNRPINGNGKQ-----
Oranutan   EHVNLPINGNGKQ-----
Macaque    EHVNLPINGNGKQ-----
Human      EYVNLPINGNGKQ-----
Bovine     EHMNRPINGNGKQ-----
Goat       EHVNRPINGNGKQ-----
Pig        EHVNRPINGNGKN-----
Toad       AHKKRPINGNGKQQ-----
Zebrafish  NFKKLPINGNGKQ-----
Salmon     AYKKLPINGNGKPVII LNKEG
Mussel     HFKNMPVNGNGKQ-----
Clam       EFKNMPVNGNGKQ-----
Wuchereria NRAKIPVNGNGKQ-----
bancrofti  : *:*****

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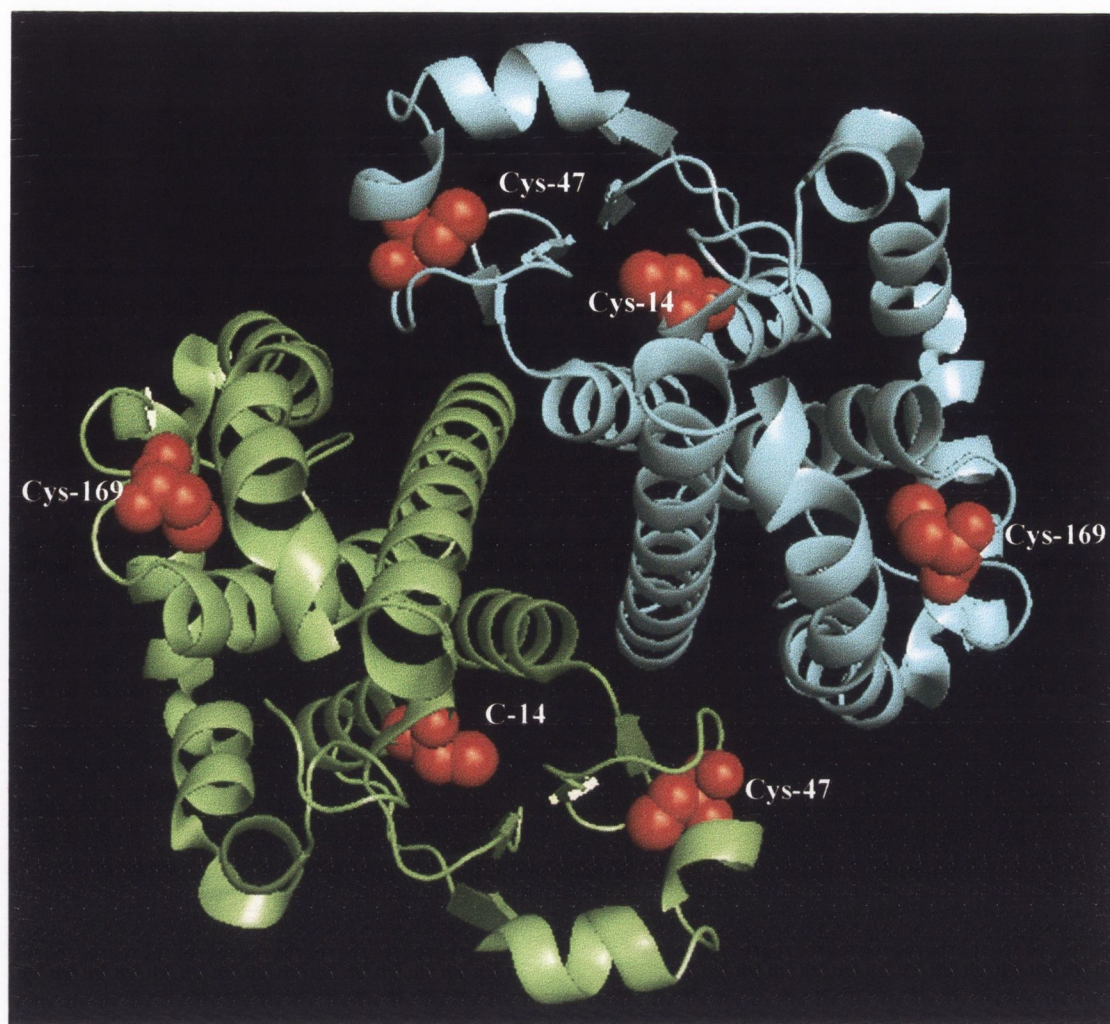
**Figure 6.2 Clustal alignment of GSTP1-1 from various species**

A clustal alignment was done on GSTP1-1 sequences found from a blast search of mGSTP1-1. The cysteines were highlighted in red and lysine 54 is highlighted in blue. Cys-169 is the only cysteine found in all species looked at.

in Fig. 6.3, which depicts the mGSTP1-1 dimer where the density of glutathione is removed. Here, the two subunits are shown with the active sites facing away from each other, with the Cys-14 and Cys-47 on Domain I of one subunit and the Cys-169 on Domain II of the other subunit exposed at the surface.

The main interest in GST-Pi thiols was originally focussed on Cys-47 as it was shown to be the residue that was alkylated on treatment with N-ethylmaleimide (NEM) and this was clearly associated with NEM inactivation (Tamai *et al.*, 1990). Ricci *et al.* (1991) showed that hGSTP1-1 undergoes an oxidative inactivation due to the formation of an intrachain disulfide bond. This disulfide bond was shown to be between Cys-47 and Cys-101. It forms under alkaline conditions and requires traces of metal ions. Using non-reducing SDS-PAGE, Ricci *et al.* (1991) showed that no interchain disulfide bonds are formed. This correlates with the inactivation of the enzyme. However, it is unclear if the disulfide bond forms before or after the enzyme is treated with SDS, which will unfold the protein and may be required for disulfide formation. It is possible that, in buffer, the thiol of Cys-47 is oxidized (to sulfenic acid?) and that, after the protein unfolds in SDS, the remaining thiol of Cys-101 displaces  $\text{OH}^-$  to form the disulfide bond. The experimental observation that inactivation parallels the appearance of a subunit with altered mobility suggests that the disulfide may be present before the addition of SDS. The activity was recovered by treating the inactivated enzyme with 4 mM DTT for 10 min at pH 8 and 37°C. Hydrogen peroxide was used to identify the cysteine residues involved in disulfide formation in human and rat GSTP1-1 (Shen *et al.*, 1993). The four cysteines in the rGSTP1-1 were mutated to alanine and the inactivation with hydrogen peroxide was examined. C101A was found to be insensitive to hydrogen-peroxide treatment but

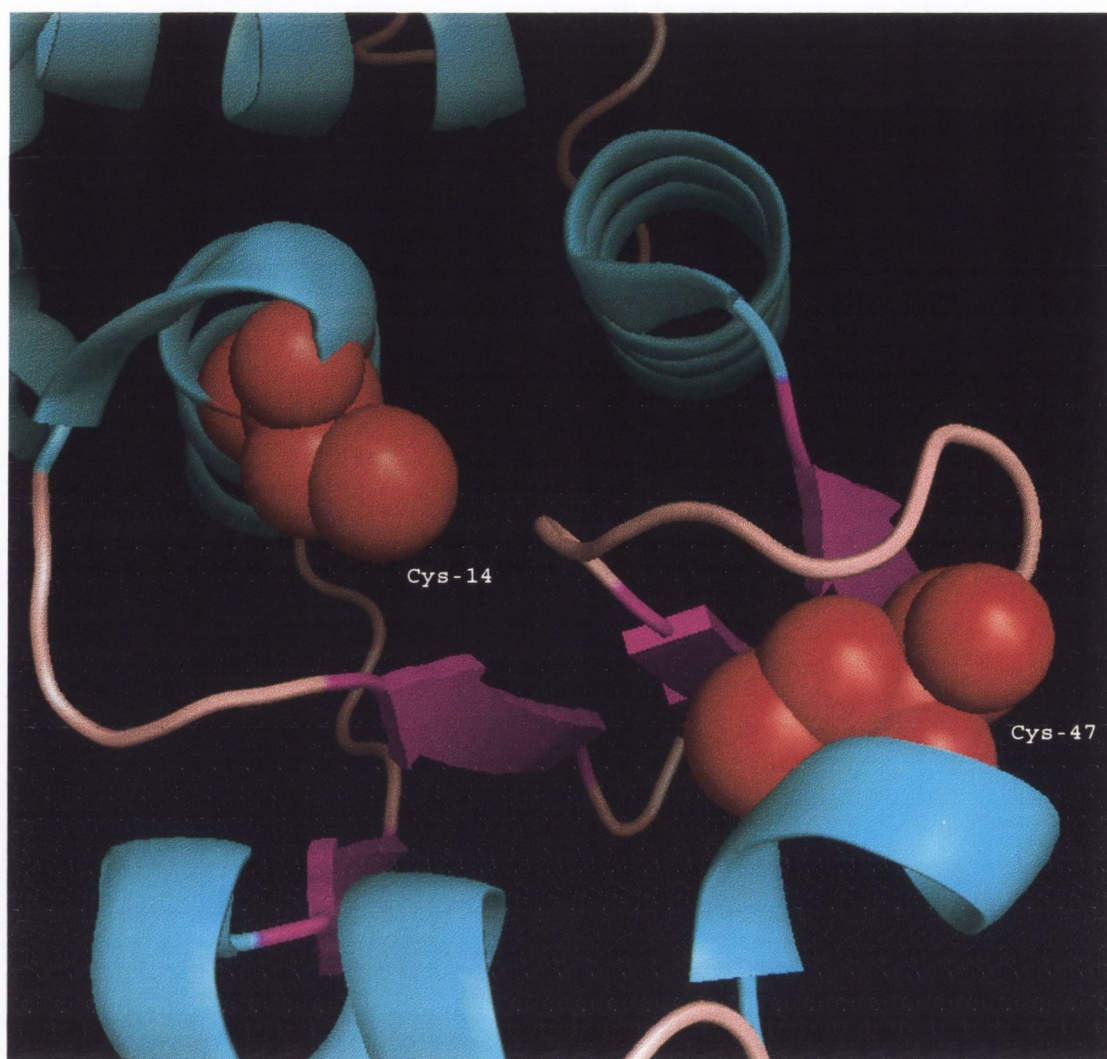




**Fig 6.3 Ribbon structure of mGSTP1-1 dimer**

The two subunits of mGSTP1-1 are shown in different colours with the cysteines shown as spheres in red. The density of the substrate has been removed.



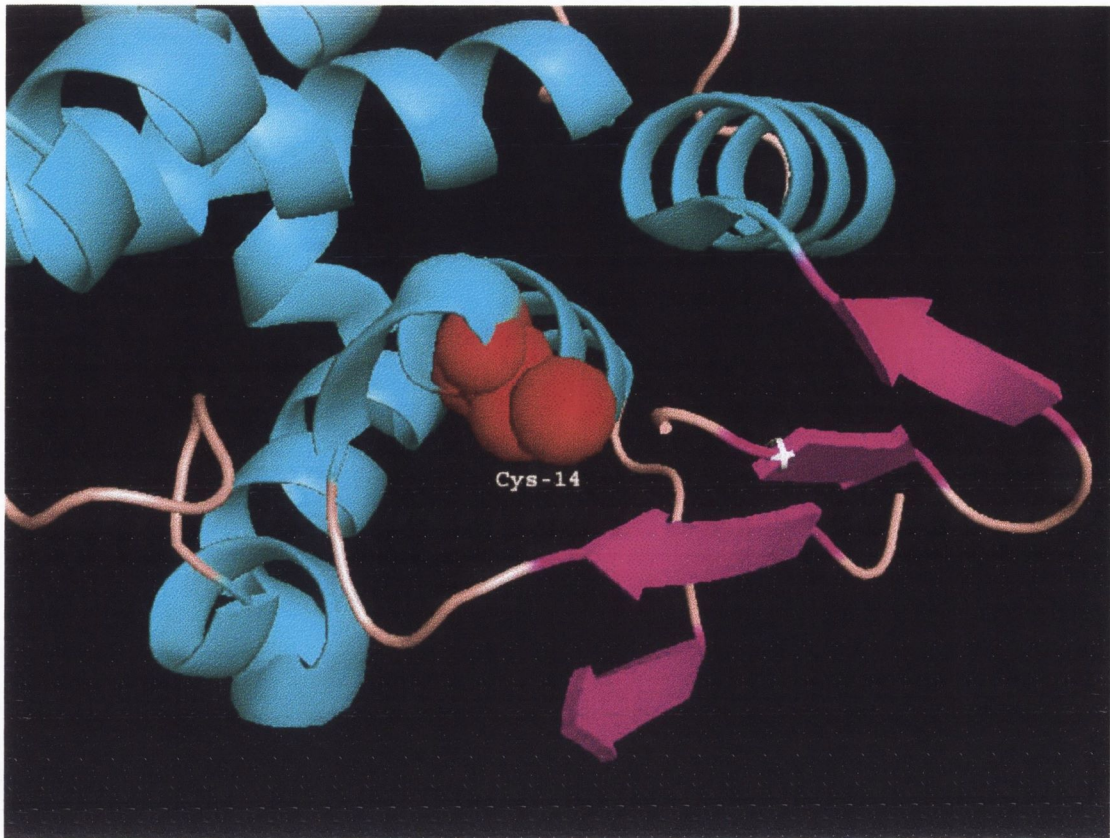


**Fig 6.4 Ribbon structure of floppy loop**

Ribbon structure of the  $\alpha 2$  loop and G site of mGSTP1-1 with the density glutathione removed showing the position of Cys-14 and Cys-47, which are shown in red spheres.

C14A and C169A are much more sensitive to hydrogen peroxide treatment than the native enzyme. Shen et al. (1993) suggested that disulfide bonds between Cys-47 and Cys-101 normally form on oxidation to give an intrachain disulfide bond. This is in spite of the fact that these residues are 18.4 Å apart in the crystal structure, indicating that the  $\alpha 2$  loop is very flexible. In the case of mGSTP1-1, which has no Cys-101, the native enzyme is inactivated by hydrogen peroxide and the C14A and C169A mutants have the same residual activity (3%) after hydrogen-peroxide treatment. Inactivation studies using ethacrynic acid with mGSTP1-1 showed similar rates of inactivation for the native enzyme and the C169A mutant but a more rapid inactivation of the C14A mutant (Maloney, unpublished observation). Flexibility in the  $\alpha 2$  loop can also be seen in the crystal structure of the hGSTP1-1 apo-enzyme (Oakley *et al.*, 1998) and the mGSTP1-1 carboxymethylated enzyme (Vega *et al.*, 1997), where there is no density for the  $\alpha 2$  loop. In the case of the human apo-enzyme, there is no density for residues 36-48 whereas, for the carboxymethylated mouse enzyme, it is residues 35-51 that have no density. In mGSTP1-1, which lacks Cys-101, there is the possibility that the Cys-47 forms a disulfide bond with Cys-14. In the structure with bound substrate this does not look possible, as shown in Fig. 6.4. However, if the residues that have no density in the carboxymethylated mGSTP1-1 structure (Fig. 6.5) are removed, the Cys-14 is more exposed and Cys-47, which will now be on an unstructured  $\alpha 2$  loop, may be able to form a disulfide bond with Cys-14. Studies on the extent of reaction of DTNB with the native and mutant enzyme clearly showed that a comparison of the sum of the release of nitrothiobenzoate (NTB) for the single thiol of the C14\* and C47\* mutants is less than the extent of the reaction with C169A, where both Cys-14 and Cys-47 are present. The simplest explanation is that the formation of an initial mixed disulfide between Cys-47 and





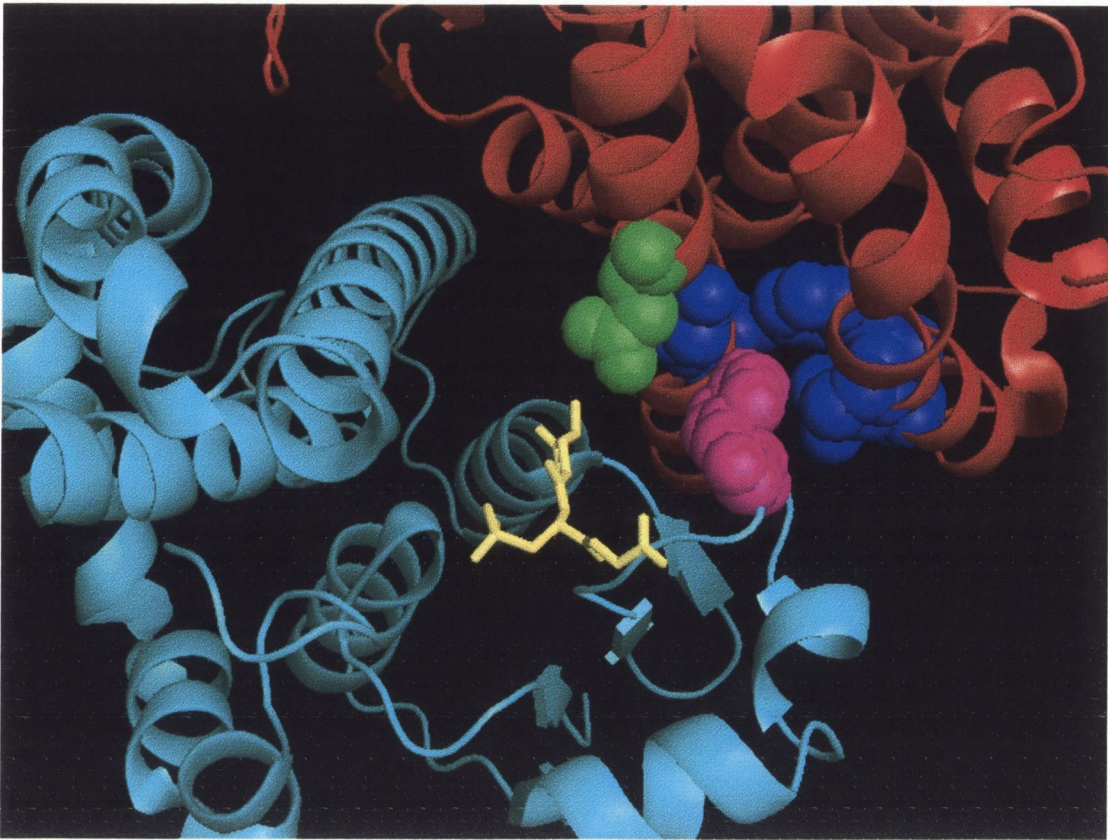
**Fig 6.5 Ribbon structure of mGSTP1-1 with the  $\alpha$ 2-loop residues missing**

Ribbon structure of Domain I of mGSTP1-1 with the residues (35-51) removed that have no density in the carboxymethylated mGSTP1-1 structure.



DTNB is followed by displacement of a second molecule of NTB by Cys-14. The formation of the initial mixed disulfide with Cys-47 would certainly destabilize the  $\alpha 2$  loop and a small extension of the  $\alpha 1$  helix with movement of Pro-53 may allow the formation of a disulfide bond between Cys-14 and Cys-47. This observation coupled with the markedly enhanced sensitivity of the C14A mutant to inactivation by ethacrynic acid (see above) strongly suggests some functional link between Cys-14 and Cys-47.

The kinetic independence of the subunits of GSTs has been well established (Danielson *et al.*, 1985) and steady-state kinetic pathways for hGSTP1-1 (Ivanetich *et al.*, 1989) and mGSTP1-1 (Phillips *et al.*, 1991) are consistent with a rapid equilibrium random sequential Bi Bi mechanism. In these earlier studies, good fits to hyperbolic expressions were obtained, as was the case in the current study using the native wild type enzyme. However even at low concentrations of CDNB, Hill coefficients of greater than one are clearly apparent indicating positive cooperativity. Indeed, for all the mutants examined in this study, there is evidence that Hill coefficients generally exceed one except in the case of the C169A mutant, where it is slightly less than one. A degree of caution must be exercised since criteria for goodness of fit must be considered carefully. More complex rate equations will generally fit better due to the increased flexibility that the additional coefficients allow. In several instances (e.g. C14A and C169A) where apparent ping-pong kinetics are observed, the fits to hyperbolae are similar in terms of the distribution of residuals as are the fits to the Hill equation. However, most GSTP1-1 mutants display some degree of cooperativity. Although the active sites are at opposite ends of the dimer, there are hints that there may be some communication between them. Ricci *et al.*



**Fig 6.6 Ribbon structure of mGSTP1-1 lock and key motif with Asp-98**

Glutathione is represented in yellow and Tyr-49 is pink on one subunit. The five amino acids shown in blue are those that Tyr-49 is in contact with on the opposite subunit, (Met-91, Gly-95, Pro-128, Phe-129 and Leu-132). Asp-98 is shown in green. The subunits are in different colours.



(1995) showed that mutating the Cys-47 in hGSTP1-1 to an alanine decreases the affinity for glutathione and triggers positive cooperativity with respect to this substrate. They also showed the importance of Lys-54 in forming an ion pair with Cys-47, which appears to stabilize the  $\alpha 2$  loop. Studies of mGSTP1-1 in complex with *S*-hexylglutathione revealed that Cys-47 is located on the surface with its thiol group pointing into a small hydrophobic pocket, formed by some main chain atoms of Lys-44 and Gln-51 and the side chain atoms of Trp-38 and Leu-52. The same residues are involved on the opposite side of the  $\alpha 2$  loop in the binding of glutathione and this may in part explain why chemical modification of Cys-47 disturbs the binding of glutathione. It is clear that, in the presence of substrate or glutathione *S*-conjugates, Cys-47 is less accessible, suggesting that ligand binding to the G-site stabilizes a distinct form where Cys-47 is cryptic.

Limited proteolysis with trypsin, which cleaves the bond at Lys-44 in the  $\alpha 2$  loop, has been used as a measure of flexibility of this structure as it is more prone to digestion in an exposed state than when it is locked down with substrate present (Lo Bello *et al.*, 1993b). Increased proteolysis was seen in enzyme with mutated Cys-47 and Lys-54 groups, showing their importance in stabilizing the  $\alpha 2$  loop.

There are residues involved in subunit interactions that may be implicated in communication between the active sites. A lock-and-key structure is a common feature of GSTs of the Alpha, Mu and Pi classes. The key residue in GSTP1-1 is a tyrosine at position 49 on one subunit, which is wedged into a lock on the other subunit formed by five residues on helices  $\alpha 4$  and  $\alpha 5$  in Domain II (Fig 6.6). These residues are Met-91, Gly-95, Pro-128, Phe-129 and Leu-132. When glutathione is present, Tyr-49 makes 15 van der Waal and hydrogen-bond connections with the opposite subunit, while in the apo-enzyme, there are only eight such contacts.



Mutation of Tyr-49 to leucine or to threonine resulted in a decrease of activity to less than 1%. There was also a significant deviation from the hyperbolic substrate dependence of the native enzyme to a sigmoidal curve (Stenberg *et al.*, 2000). This cooperativity is thought to be due to the stabilization of the structure at one subunit by the binding of glutathione, coupled with increased affinity for glutathione in the other subunit. The free enzyme is a substrate for chymotrypsin and the cleavage site is between residues Tyr-49 and Gly-50 (Martini *et al.*, 1993). This was used to show the flexibility of Tyr-49 when glutathione or glutathione *S*-conjugates are not bound to the G-site. Another important residue is Asp-98, which is the only residue from the other subunit ( $\alpha 4$  helix) to make contact with glutathione. Mutating this residue (D98) to a leucine causes almost complete loss of activity and less than 6 % of binding to glutathione remains (Manoharan *et al.*, 1992). More recent work (Hegazy *et al.*, 2004; Misquitta *et al.*, 2005) suggests that a chain of water molecules may relay information between the two sites of the dimer.

An important part of the catalytic mechanism is the stabilization of  $\text{GS}^-$  in the active site and the main residue responsible for this is Tyr-7. Its hydroxy group forms a hydrogen bond with the sulfur atom of glutathione and probably stabilizes and orientates the thiolate in a productive fashion. It has been argued that this residue could act as a general base, accepting the proton released by the thiol group (Atkins *et al.*, 1993). Garcia-Saez *et al.* (1994) suggested that, if Tyr-7 was in its ionized form, a positively charged residue could be in closer proximity to it in the free enzyme and thus be removed when glutathione binds. The structure of the unliganded Cys-47-modified enzyme, which shows Tyr-7 in an unmodified environment, proves that this is not the case (Vega *et al.*, 1998). The Tyr-7 hydroxy group appears to be

protonated, accepting a hydrogen bond from the main chain NH group of Arg-13 and donating a hydrogen bond to a well-defined water molecule, W0, which occupies the thiol position in the absence of glutathione.

It seems clear from recent work on hGSTP1-1 that the proton removed from GSH does not remain bound to the enzyme but is extruded into the solvent in a step that appears not to be rate-limiting (Caccuri *et al.*, 1998). Several water molecules occupy strategic positions in the active site and provide the requirements for hydrogen bonding to glutathione. In particular, W1, W2 and W4 are found at hydrogen bond distance from the thiol group of glutathione. The net of water molecules seems very stable and is seen in both the unliganded Cys-47 carboxymethylated enzyme and the mGSTP1-1:GSH complex. In the unliganded structure, one additional water molecule is observed, W0, which is located in a position equivalent to the thiol group of glutathione (Vega *et al.*, 1998). In the structure of mGSTP1-1 in complex with *S*-(*p*-nitrobenzyl)glutathione, W4 is no longer present, as this water molecule is displaced during binding of the hydrophobic substrate. This gives a picture of the active site at different points during the reaction. Molecular dynamic studies have suggested that GS<sup>-</sup> is stabilized by the OH group of Tyr-7 and the water molecules W1, W2 and W4, and suggests that the proton from glutathione may leave on the water molecule W0 (Orozco *et al.*, 1997). The structure of mGSTP1-1 with the Cys-47 changed to an alanine complexed with *S*-(*p*-nitrobenzyl)glutathione has been solved (Costa, Coll, Walsh and Mantle unpublished) and there is an intriguing difference in the alignment of the inhibitor at the two sites. When the two sites are superimposed, the inhibitor clearly does not bind symmetrically as it does in the two essentially equivalent subunits of the native enzyme (Fig. 6.6). In the case of the mutant C47A crystallographic subunit A, the glutathione adduct adopts the same conformation as



seen in both subunits of the native enzyme. However, in crystallographic subunit B, the *p*-nitrobenzyl moiety is clearly rotated. This heterogeneity in the two subunits has implications for the observed cooperativity but, more interestingly, there is another major consequence: in crystallographic subunit A, W0 is clearly present while it is absent in crystallographic subunit B. Crystallographic subunit A therefore represents a “snapshot” before glutathione has fully bound and where W0 is still bound to Tyr-7. A steered molecular dynamic study where this structure is used as a starting point and where the *p*-nitrobenzyl moiety is removed to leave glutathione will be conducted shortly and it is hoped that this will provide evidence that W0 exits with the glutathione proton.

The kinetics of the cysteine-to-alanine mutants of mGSTP1-1 were studied for all the combinations, C14A, C47A, C169A, C14\*, C47\* C169\* and 3A. From previous work on human and rat GSTP1-1, it was expected that the Cys-47 mutants would give the greatest deviation from the kinetics of the native enzyme. Kinetic studies conducted on hGSTP1-1 for cysteine-to-serine mutants showed a 25% decrease in  $k_{\text{cat}}$  for the C14S mutant while the replacement of Cys-169 by serine scarcely affected the kinetic parameters (Kong *et al.*, 1991). The C47S mutant had a similar  $k_{\text{cat}}$  to the native enzyme but a five-fold increase in  $K_{\text{m}}^{\text{GSH}}$ . Although Cys-14 makes no contacts with glutathione, it is in the back of the active site and its replacement by alanine might be predicted to result in modest effects on kinetic parameters. Cys-169 is far away from the active site and has a high  $\text{p}K_{\text{a}}$ , which makes it unreactive at neutral pH. As a result, few changes in the kinetic parameters were anticipated on mutation of this residue to alanine. However, a preliminary examination of the sequence alignment in Fig. 6.2 shows that the only conserved



cysteine is Cys-169, which is even found in parasite GSTP1-1. This suggests that it may play an important functional role. It was therefore surprising that, while several Cys-to-Ala mutations revealed cooperativity with glutathione as the variable substrate, it was the C14A and C169A mutants that appeared to result in a complete change in kinetic mechanism. The primary plots of initial rates versus variable substrate concentration show a sharply contrasting pattern for these two mutants (C14A and C169A) when compared with all other mutants and the wild-type enzyme. At low substrate concentration, the slope ( $V/K$ ) is clearly not dependent on the “fixed” substrate concentration. This was confirmed by graphing the results as double reciprocal plots, which resulted in families of parallel lines. Care has to be taken to ensure that the observation is not simply due to a low  $K_{ia}K_b$  term, however, neither the  $K_{ia}$  (the dissociation constant for glutathione; see Table 4.3) nor the  $K_b$  (the Michaelis constant for CDNB; see Table 4.2) was changed significantly. Segal (1975) has discussed a number of other possibilities that can result in apparently parallel plots for a non-ping-pong enzyme. However, a number of observations are consistent with ping-pong kinetics for these two mutants. There is little doubt that glutathione and the second substrate (CDNB) bind to the enzyme in an apparent ternary complex. These two subsites (G and H sites) are clearly seen in all GST structures solved to date and most GSTs exhibit non-parallel primary plots, which is consistent with a kinetically significant ternary complex. However, there is also increasing evidence that the forgotten product (the glutathione derived proton  $H^+$ ) is released quantifiably from the enzyme-glutathione complex (Caccuri *et al.*, 1998; Caccuri *et al.*, 1999) to form an enzyme-bound thiolate complex (E.GS<sup>-</sup>). In addition to measuring proton extrusion through pH change, the thiolate anion is clearly seen at 239 nm in the case of rat

microsomal glutathione transferase 1 (Armstrong *et al.*, 2004) and human Pi (Caccuri *et al.*, 1998).

For this proton release step to give ping-pong kinetics, the only requirement is that this is an irreversible step. The derived rate equation (see Scheme 4.2) then predicts ping-pong patterns. Why do these two mutants behave this way when, for all other cysteine to alanine mutants and the wild type enzyme, the proton release step is clearly in equilibrium? It is tempting to speculate that a similar situation may exist for mGSTP1-1, as has been suggested for the E1 component of pyruvate dehydrogenase (Frank *et al.*, 2004). In this case, proton production at one site is suggested to be passed down a 'proton wire' using a Grotthus-like mechanism (Latimer *et al.*, 1920) to fill a proton need at the other site. The water channel identified (Hegazy *et al.*, 2004; Misquitta *et al.*, 2005) may be such a candidate although it is difficult to define what the reciprocating steps may be in the case of mGSTP1-1. In fact, there have been a number of suggestions as to the ultimate proton sink, including: a channel filled by four water molecules, which passes between helices  $\alpha A$  and  $\alpha F$  and reaches the solvent at Arg-11 (Garcia-Saez *et al.*, 1994); the  $\alpha$ -carboxylate of the  $\gamma$ -glutamyl residue of GSH (Widersten *et al.*, 1996) or simply the extrusion of W0 (Parraga *et al.*, 1998).

Glutathionylation, the formation of a mixed disulfide between a protein thiol and GSH, has been shown to be important in both signalling and protection. Glutathionylation of thiols that have been oxidized to sulfenic acid prevents further irreversible oxidation and, when stress levels decrease, the glutathione can be removed by thioredoxin, glutaredoxin or small thiols such as GSH. Glutathionylation has also been shown to be important in regulating the effects of protein function and



plays a role in determining the response of many cellular pathways to oxidative stress. These include its effects on various protein tyrosine phosphatases (PTPs) such as Phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which regulates phosphatidylinositol 3,4,5-trisphosphate and the Akt/protein kinase B signalling pathway, both of which are important in cell-cycle progression and cell survival (Sun *et al.*, 1999); its role in metabolism where it inactivates GAPDH (Shenton *et al.*, 2003) to shunt glucose equivalents into the pentose-phosphate pathway for the generation of NADPH; and the effect on various MAP kinase pathways, including the inhibition of MEKK1 (Cross *et al.*, 2004) and the activation of ASK1 (Tobiume *et al.*, 2002).

Originally, oxidative stress was seen as a by product of metabolism in the mitochondria or as part of an immune response where macrophages and neutrophils produce large amounts of  $\text{H}_2\text{O}_2$  during the oxidative burst reaction with NADPH oxidase (Babior *et al.*, 2002). However, new data show that proteases play a more important role than the reactive oxygen species (ROS) as antibacterial agents (Reeves *et al.*, 2002). ROS are now being recognized as part of the cells signalling function, with non-phagocytic NADPH oxidase being found in many types of cells (Li *et al.*, 2003) and mitochondria being shown to produce  $\text{H}_2\text{O}_2$  after glutathionylation of complex 1 (Finkel *et al.*, 2000).  $\text{H}_2\text{O}_2$  is a small diffusible, and ubiquitous molecule that can be synthesized, as well as destroyed, rapidly in response to external stimuli. As such it fulfils the prerequisites for an intracellular messenger. It has been shown to be the major ROS species in cells activated by, transforming growth factor- $\beta$ 1 (Ohba *et al.*, 1994), platelet derived growth factor (PDGF; Sundaesan *et al.*, 1995) and epidermal growth factor (EGF; Bae *et al.*, 1997). Most cysteine residues in proteins have a  $\text{pK}_a$  value of 8.5, and therefore do not exist as anions at physiological pH



values. If, however, the cysteine residue is located in the vicinity of a positively charged amino acid, its  $pK_a$  value can be lowered to a  $pK_a$  below 5.0. Such a cysteine is deprotonated at physiological pH and becomes a target for the oxidizing action of  $H_2O_2$ . The fact that only certain proteins inside the cell carry an oxidizable cysteine at a critical position is the reason why such a small molecule as  $H_2O_2$  can act as specific second messenger.

An understanding of how this happens and a method of directed glutathionylation would be useful in order to study this process. The oxidation states of thiols and the formation of disulfide bonds are in a state of equilibrium in a cell, which makes it hard to quantify exactly what is happening. mGSTP1-1, with its three thiols in different environments each with different reactivity, provided a good model for the study of glutathionylation. Being able to mutate each cysteine, either alone or in combination, allowed the influence of each cysteine to be determined. In addition this facilitated a more detailed study of the reactivity of the three cysteines of mGSTP1-1.

When a method was being devised to demonstrate glutathionylation of a protein thiol using the novel reagent GSSN, problems were encountered because of the presence of multiple reactive thiols. Original experiments carried out with native mGSTP1-1 showed release of NTB as if the glutathione from GSSN had formed a disulfide bond with the cysteine residues on the protein. However, when radiolabelled glutathione was used, it was found that this was not the case, as less than 5% of the glutathione was bound to the protein. By reacting a protein thiol with GSSN to form a mixed disulfide bond with glutathione, this disulfide became the target of other reactive thiols, which would then release the glutathione with concomitant formation of a protein–protein disulfide bond or an internal disulfide involving a single subunit.

The experiments were repeated with C47\* to reduce this effect and the amount of bound glutathione increased to 50%. Even with only one cysteine per monomer, there is still some protein–protein disulfide-bond formation and a possible explanation for this is given in Scheme 5.1. However, a more interesting result was obtained when the same experiment was repeated with C14A, which was shown to have roughly the same amount of bound glutathione as the single cysteine C47\*. This suggests that Cys-14 plays an important role in the removal of glutathione from the enzyme after it has been treated with GSSN. The C169A mutant that has both Cys-14 and Cys-47 behaves in a similar manner to the native enzyme losing the bulk of the bound glutathione.

The present study has identified the “alkaline thiol” of mGSTP1 originally described by Phillips and Mantle (1993) as Cys-169. In addition Cys-47 is confirmed as the reactive “neutral” pK thiol. Cysteine-14 in the C14\* mutant is unreactive at neutral pH and only slowly reacts under alkaline conditions with only a gradual increase to 0.3 mol of NTB release per mol of monomer and although it is still increasing it is not possible to get an accurate value as the reaction has shown to be unstable over time periods greater than 30 min (when Cys-169 has gone to completion in 15 min with approximately 1 mol of NTB released per mol of monomer at alkaline pH). Several key experiments however suggest that Cys-14 can form a disulphide with Cys-47 and this may occur under conditions when the basic dimeric structure is maintained. A comparison of the extent of reaction of DTNB with GSTP1 forms with only Cys-14 or only Cys-47 sum to a significantly lower value than obtained when both Cys-14 and Cys-47 are present (e.g. the wild type and the C169A mutant). A simple mechanism to explain this has been presented where an initial mixed disulphide between NTB and Cys-47 results in destabilising the  $\alpha$ 2-loop allowing



Cys-14 to attack the modified Cys-47, releasing more NTB than would be expected and forming a stable disulphide between Cys-14 and Cys-47. Whether this thioredoxin like half reaction has any functional significance is presently unclear, however it should be noted that treatment of C169A with relatively low concentrations of  $H_2O_2$  resulted in the formation of the Cys-14/Cys-47 disulphide as revealed by a species running with an apparent mobility of 20kDa on SDS/PAGE in the absence of any mercaptoethanol.

Advantage has been taken of the availability of the cysteine to alanine mutants of GSTP1 to develop a reagent to study glutathionylation reactions *in vitro*. A simple procedure has been developed to isolate GSSN, a novel reagent that clearly glutathionylates the reactive thiol (Cys-47) of GSTP1. This reaction is monitored by the release of the yellow reporter NTB which allows the reaction to be followed spectrophotometrically. The synthesis of  $G[^{35}S]SN$  has allowed the fate of the glutathione moiety to be followed more easily. During early work it became clear that the additional thiols of native mGSTP1 clearly displaced the glutathione, bound initially to Cys-47, as GSSG. In preliminary experiments it was argued that this displacement reaction may have been due to the less reactive but accessible cysteine-169. However the retention of radiolabelled glutathione by the C169A mutant was only slightly improved when compared with the wild type. Stable glutathionylation was achieved with C47\*, where only one thiol (with a low pK) is expressed. Intriguingly the C14A mutant also retained approximately 0.5 mole glutathione per mole of subunit, further evidence that Cys-14 can react with Cys-47 when it is modified to a mixed disulphide. This work has implications for the current interest in glutathionylation as a signalling mechanism. It is clear that glutathionylation of



proteins does occur and that this is mediated by two major mechanisms. The first involves disulphide interchange between GSSG, formed as a result of oxidative stress, and protein thiols. The second involves specific oxidation of particular thiols to sulphenic acids and the subsequent reaction of these modified cysteines with reduced glutathione. What has been intriguing in several studies of redox stress is that a limited number of proteins are reported as “stably” glutathionylated – these include almost invariably carbonic anhydrase (Mallis *et al.*, 2000), GAPDH (Lind *et al.*, 1998), phosphorylase (Park *et al.*, 1988) and actin (Wang *et al.*, 2001). It is clear from the present work that glutathionylation is a facile reaction and equally that the displacement of this residue by other thiols is equally facile. In the case of at least one of the proteins reported as being “stably glutathionylated”, carbonic anhydrase, a crystal structure has been solved showing the glutathione moiety docked onto the protein surface. It may be that of all the proteins glutathionylated under harsh conditions of redox stress only a fraction are resistant to thiol-mediated displacement and these may dock the glutathione in an unreactive conformation.

Kinetic studies of all the cysteine to alanine mutants have revealed two surprising features. Firstly, cooperative binding of glutathione is the normal behaviour, suggesting that the wild type may have been selected for suppressing this intrinsic tendency. Secondly two mutants exhibit apparently ping-pong kinetics. This may be explained if the proton release step is irreversible, although other explanations are possible. Several lines of evidence suggest that a crucial water molecule, W0, may be the first acceptor of the GSH proton. How the release of this solvent molecule may become essentially irreversible for the C14A and C169A mutants will require

further work but may be related to the water molecules currently being suggested as mediating interaction between the two sites of the GSTP1 dimer.

The present study has included an in depth analysis of the three cysteine residues of mGSTP1-1. From the study it appears that protein cysteine side chains have a role in both the function of proteins as well as a postulated role in signalling pathways in cells and a more detailed understanding of their importance in both roles is needed. It is important to study the reactions of protein thiols with GSSN and other reagents that react with thiols to get a better understanding of the dynamics of these reactions. Also a more detailed look at the protein thiols using mass spectroscopy may help to give a better understanding of the oxidation state of thiols in different local environments.

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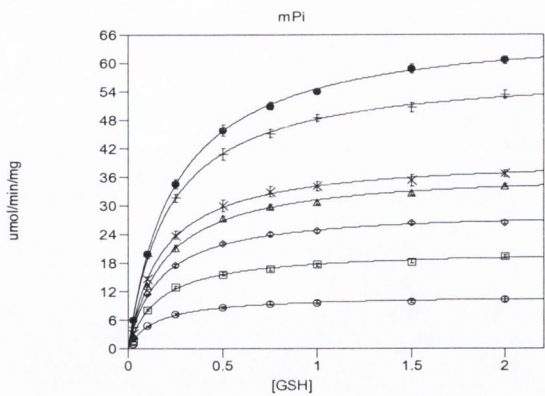
## Appendix I

This appendix contains the initial rate data for native mGSTP1 and the C14A, C47A, C169A, C14\*, C47\*, C169\* and AAA mutants. The data is shown in two formats, on one page the data is presented and fitted as hyperbolae (this includes the apparent  $K_m$  and apparent  $V_{max}$  plotted against the fixed substrate) and the alternative format is double reciprocal plots and the corresponding slope and intercept replots. Also included are fits of the Hill equation for all the mutants at fixed CDNB.

mPi

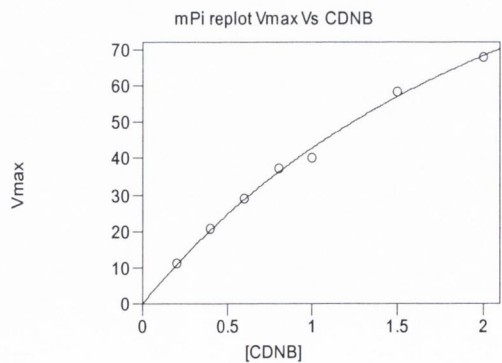
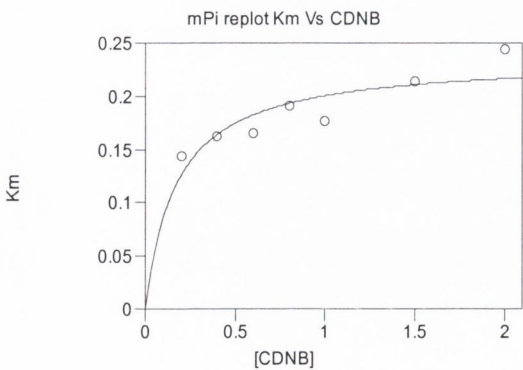
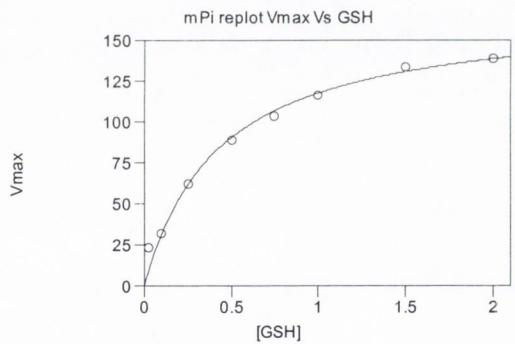
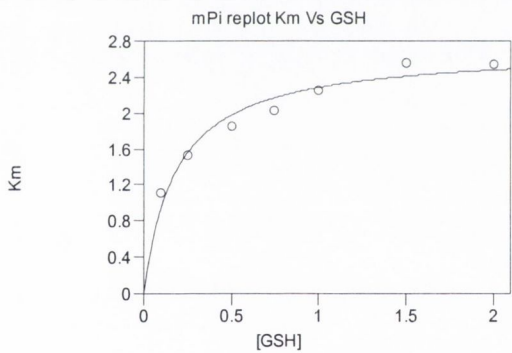
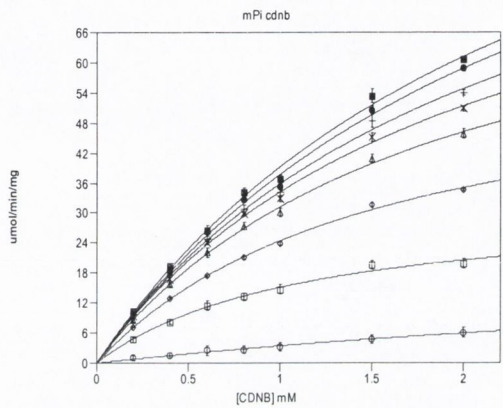
Varying GSH at constant CDNB

| [CDNB] mM | Km           | Vmax         |
|-----------|--------------|--------------|
| 0.2       | 0.144 ±0.013 | 11.099 ±0.22 |
| 0.4       | 0.162 ±0.016 | 20.638 ±0.47 |
| 0.6       | 0.165 ±0.013 | 28.992 ±0.52 |
| 0.8       | 0.192 ±0.014 | 37.212 ±0.68 |
| 1         | 0.176 ±0.013 | 40.111 ±0.71 |
| 1.5       | 0.214 ±0.011 | 58.465 ±0.75 |
| 2         | 0.244 ±0.005 | 67.898 ±0.35 |



Varying CDNB at constant GSH

| [GSH] mM | Km          | Vmax           |
|----------|-------------|----------------|
| 0.1      | 1.115 ±0.15 | 31.961 ±2.28   |
| 0.25     | 1.536 ±0.12 | 61.963 ±2.73   |
| 0.5      | 1.849 ±0.16 | 88.934 ±4.69   |
| 0.75     | 2.022 ±0.19 | 103.365 ±6.07  |
| 1        | 2.256 ±0.29 | 116.667 ±9.6   |
| 1.5      | 2.554 ±0.35 | 134.045 ±12.25 |
| 2        | 2.544 ±0.38 | 139.034 ±13.87 |

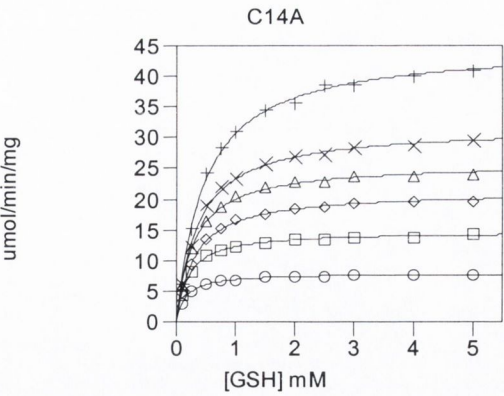


|      | GSH          | CDNB          |
|------|--------------|---------------|
| Vmax | 168.37 ±9.00 | 166.58 ±17.07 |
| Km   | 0.24 ±0.019  | 2.71 ±0.12    |

C14A

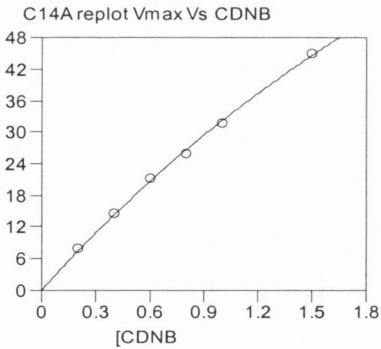
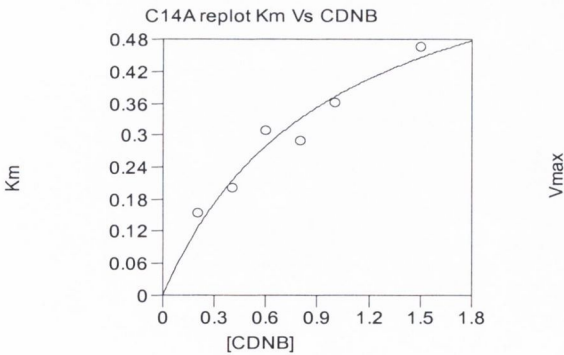
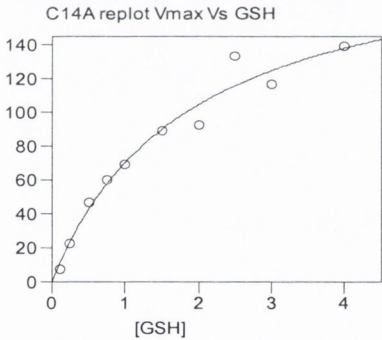
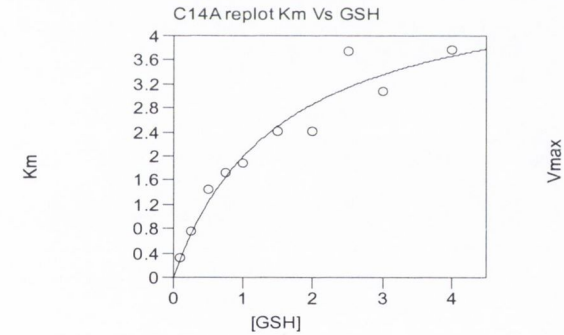
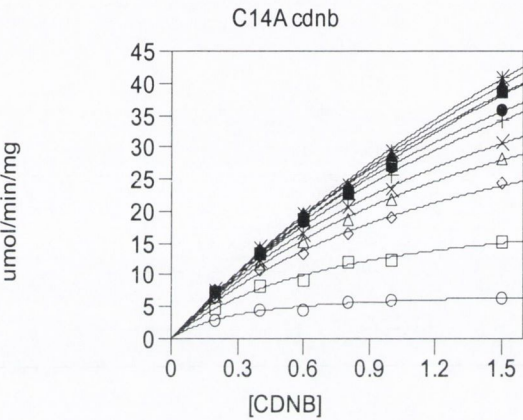
Varying GSH at constant CDNB

| [CDNB] mM | Km    |        | Vmax   |        |
|-----------|-------|--------|--------|--------|
| 0.2       | 0.155 | ±0.005 | 7.925  | ±0.046 |
| 0.4       | 0.200 | ±0.008 | 14.695 | ±0.114 |
| 0.6       | 0.309 | ±0.013 | 21.209 | ±0.198 |
| 0.8       | 0.290 | ±0.014 | 25.800 | ±0.268 |
| 1         | 0.361 | ±0.017 | 31.659 | ±0.337 |
| 1.5       | 0.466 | ±0.025 | 44.961 | ±0.608 |



Varying CDNB at constant GSH

| [GSH] mM | Km    |        | Vmax    |        |
|----------|-------|--------|---------|--------|
| 0.1      | 0.318 | ±0.071 | 7.745   | ±0.547 |
| 0.25     | 0.766 | ±0.132 | 22.680  | ±1.867 |
| 0.5      | 1.439 | ±0.196 | 46.933  | ±3.870 |
| 0.75     | 1.718 | ±0.162 | 60.062  | ±3.620 |
| 1        | 1.891 | ±0.150 | 69.091  | ±3.612 |
| 1.5      | 2.412 | ±0.206 | 88.942  | ±5.370 |
| 2        | 2.415 | ±0.144 | 92.784  | ±3.896 |
| 2.5      | 3.758 | ±0.761 | 133.443 | ±21.11 |
| 3        | 3.071 | ±0.304 | 116.813 | ±8.664 |
| 4        | 3.761 | ±0.622 | 139.137 | ±1.797 |



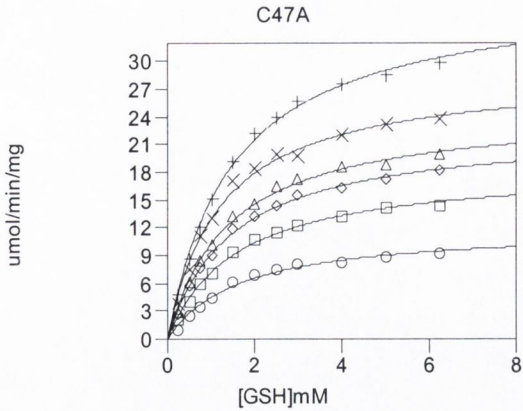
|      | GSH    |        | CDNB   |        |
|------|--------|--------|--------|--------|
| Vmax | 202.96 | ±22.12 | 195.24 | ±31.18 |
| Km   | 0.74   | ±0.146 | 5.08   | ±0.669 |



C47A

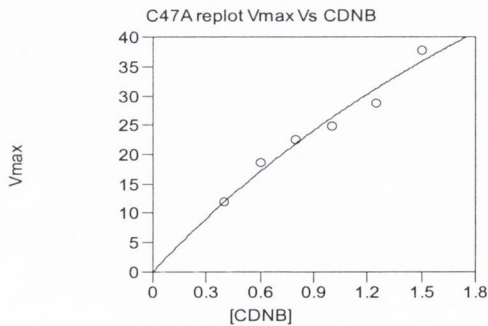
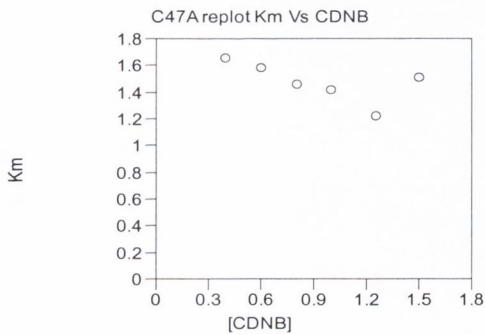
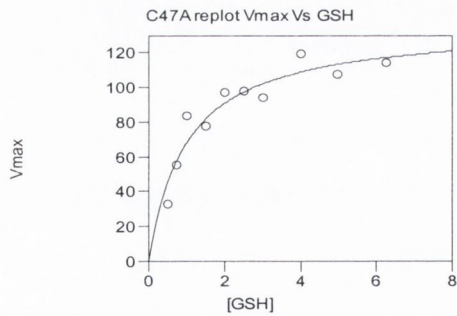
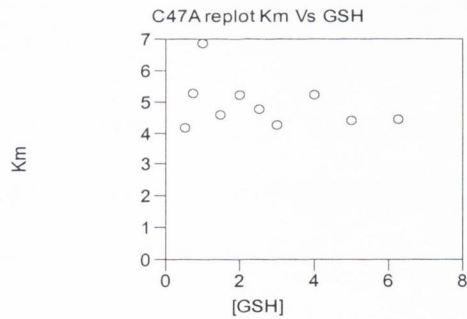
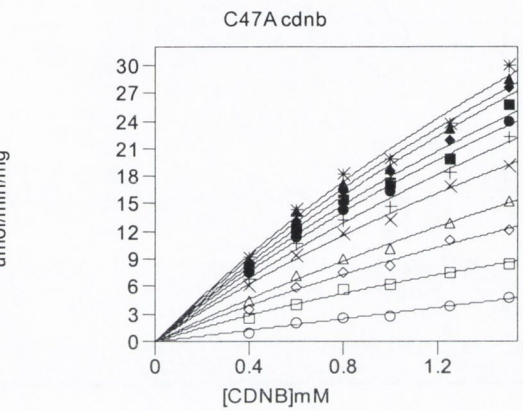
Varying GSH at constant CDNB

| [CDNB] mM | Km           | Vmax          |
|-----------|--------------|---------------|
| 0.4       | 1.652 ±0.188 | 11.974 ±0.523 |
| 0.6       | 1.578 ±0.097 | 18.531 ±0.432 |
| 0.8       | 1.463 ±0.079 | 22.586 ±0.445 |
| 1         | 1.417 ±0.104 | 24.806 ±0.658 |
| 1.25      | 1.223 ±0.102 | 28.810 ±0.818 |
| 1.5       | 1.510 ±0.085 | 37.800 ±0.783 |



Varying CDNB at constant GSH

| [GSH] mM | Km           | Vmax           |
|----------|--------------|----------------|
| 0.5      | 4.166 ±1.671 | 32.517 ±1.028  |
| 0.75     | 5.245 ±2.588 | 55.164 ±22.39  |
| 1        | 6.849 ±2.673 | 83.850 ±28.03  |
| 1.5      | 4.573 ±1.358 | 77.657 ±18.50  |
| 2        | 5.231 ±2.268 | 97.231 ±34.65  |
| 2.5      | 4.739 ±1.367 | 97.707 ±22.77  |
| 3        | 4.267 ±2.317 | 94.319 ±40.55  |
| 4        | 5.211 ±2.460 | 119.330 ±46.27 |
| 5        | 4.380 ±2.149 | 107.946 ±42.14 |
| 6.25     | 4.460 ±2.24  | 114.460 ±4.596 |

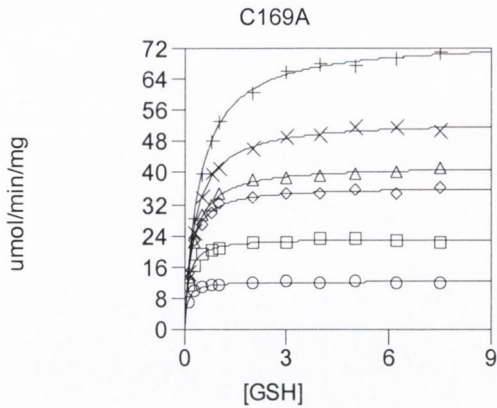


|               |               |      |
|---------------|---------------|------|
| Vmax          | GSH           | CDNB |
| 136.10 ±10.02 | 132.31 ±62.22 |      |

C169A

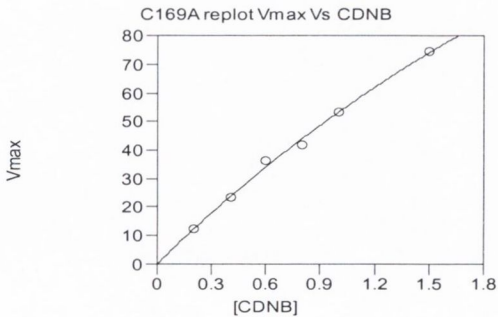
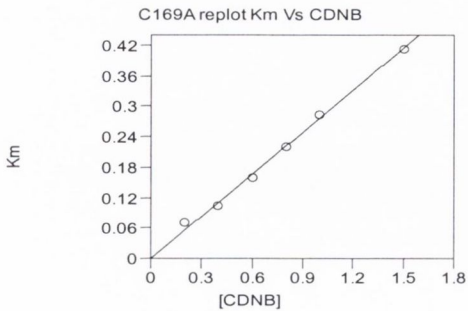
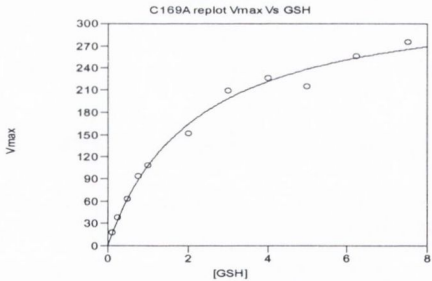
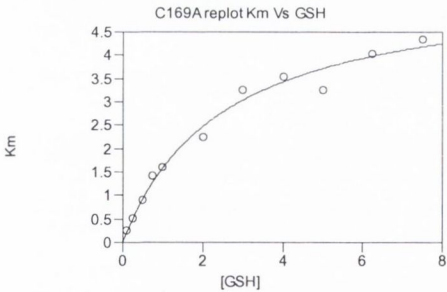
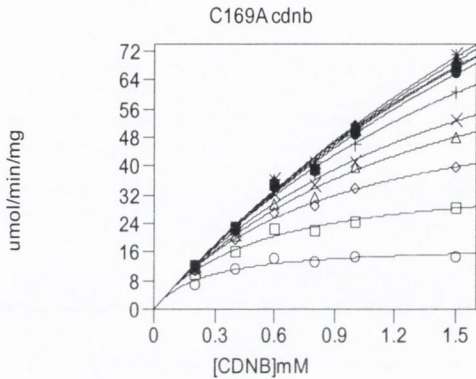
Varying GSH at constant CDNB

| [CDNB] mM | Km           | Vmax          |
|-----------|--------------|---------------|
| 0.2       | 0.072 ±0.004 | 12.330 ±0.076 |
| 0.4       | 0.103 ±0.005 | 23.290 ±0.145 |
| 0.6       | 0.159 ±0.007 | 36.483 ±0.245 |
| 0.8       | 0.221 ±0.007 | 41.821 ±0.246 |
| 1         | 0.283 ±0.009 | 53.416 ±0.311 |
| 1.5       | 0.413 ±0.011 | 74.253 ±0.418 |



Varying CDNB at constant GSH

| [GSH] mM | Km           | Vmax           |
|----------|--------------|----------------|
| 0.1      | 0.255 ±0.074 | 18.122 ±1.481  |
| 0.25     | 0.514 ±0.108 | 37.956 ±3.198  |
| 0.5      | 0.901 ±0.106 | 63.759 ±3.818  |
| 0.75     | 1.415 ±0.264 | 93.602 ±10.50  |
| 1        | 1.603 ±0.311 | 108.809 ±13.24 |
| 2        | 2.255 ±0.379 | 151.273 ±17.62 |
| 3        | 3.270 ±0.777 | 209.127 ±37.68 |
| 4        | 3.549 ±0.868 | 226.815 ±42.85 |
| 5        | 3.269 ±0.850 | 215.297 ±42.46 |
| 6.25     | 4.042 ±0.918 | 255.963 ±46.04 |
| 7.5      | 4.348 ±1.153 | 274.864 ±58.54 |

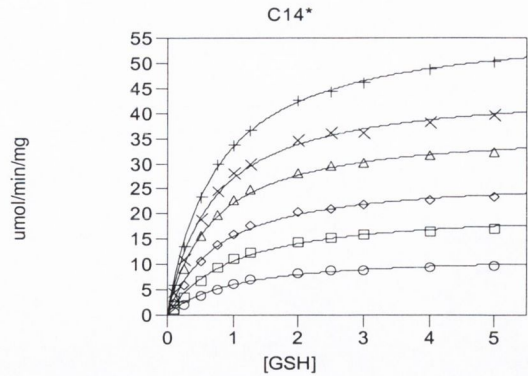


|      | GSH           | CDNB          |
|------|---------------|---------------|
| Vmax | 341.66 ±16.62 | 330.43 ±78.57 |
| Km   |               | 5.59 ±0.368   |

C14\*

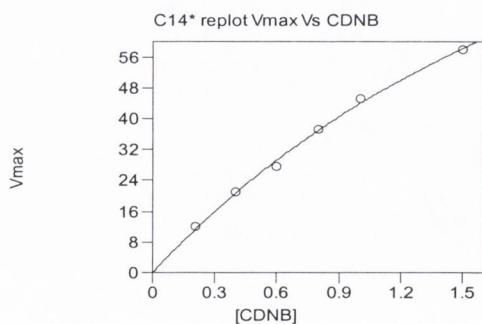
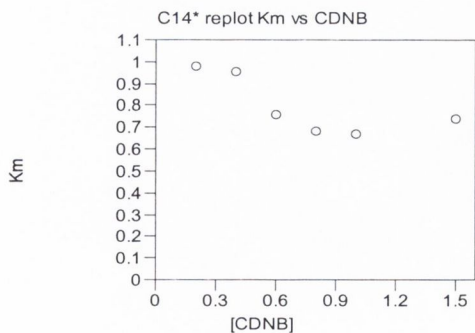
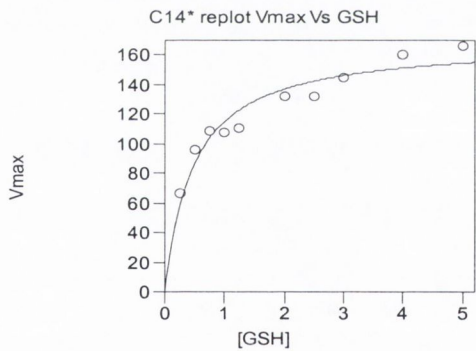
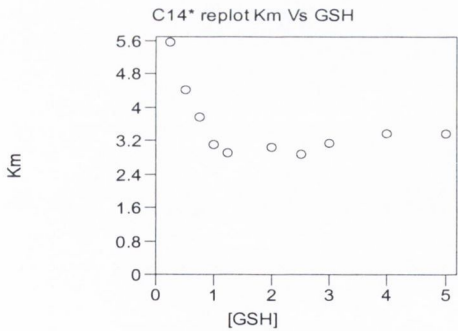
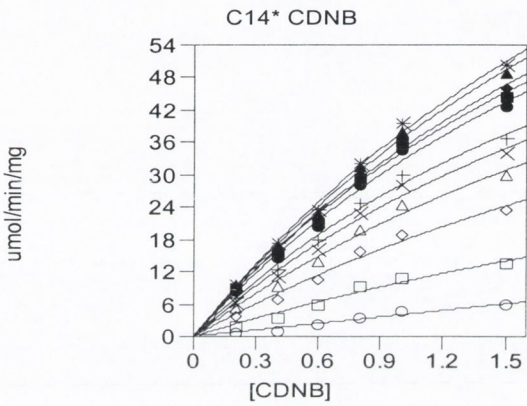
Varying GSH at constant CDNB

| [CDNB] mM | Km    |        | Vmax   |        |
|-----------|-------|--------|--------|--------|
| 0.2       | 0.981 | ±0.099 | 11.963 | ±0.410 |
| 0.4       | 0.958 | ±0.089 | 20.957 | ±0.655 |
| 0.6       | 0.760 | ±0.055 | 27.439 | ±0.611 |
| 0.8       | 0.680 | ±0.038 | 37.167 | ±0.617 |
| 1         | 0.670 | ±0.044 | 45.150 | ±0.869 |
| 1.5       | 0.737 | ±0.027 | 57.878 | ±0.657 |



Varying CDNB at constant GSH

| [GSH] mM | Km    |       | Vmax    |        |
|----------|-------|-------|---------|--------|
| 0.25     | 5.579 | ±4.8  | 66.124  | ±47.61 |
| 0.5      | 4.410 | ±2.46 | 95.437  | ±42.89 |
| 0.75     | 3.759 | ±1.57 | 108.197 | ±35.38 |
| 1        | 3.122 | ±1.22 | 107.440 | ±31.51 |
| 1.25     | 2.900 | ±0.85 | 110.144 | ±23.84 |
| 2        | 3.052 | ±0.86 | 132.034 | ±27.66 |
| 2.5      | 2.877 | ±0.85 | 132.173 | ±28.90 |
| 3        | 3.142 | ±0.65 | 144.463 | ±22.49 |
| 4        | 3.376 | ±0.77 | 160.669 | ±28.11 |
| 5        | 3.388 | ±0.78 | 166.048 | ±29.55 |



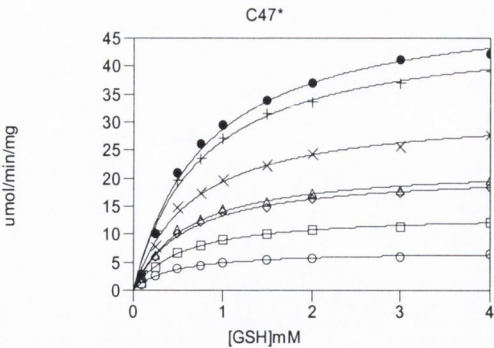
|      | GSH    |       | CDNB   |        |
|------|--------|-------|--------|--------|
| Vmax | 168.90 | ±7.60 | 170.20 | ±23.65 |



C47\*

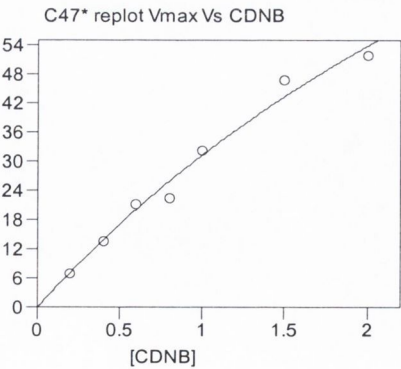
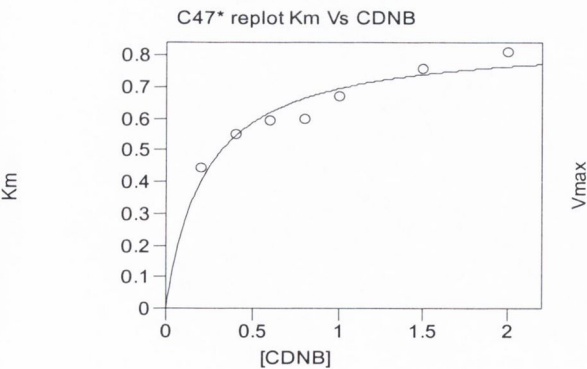
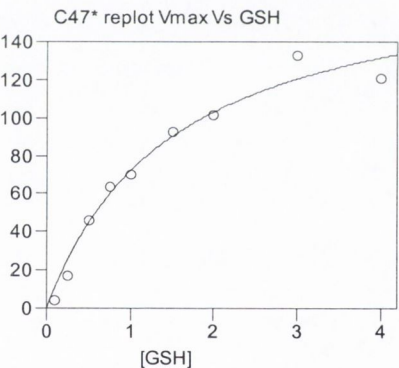
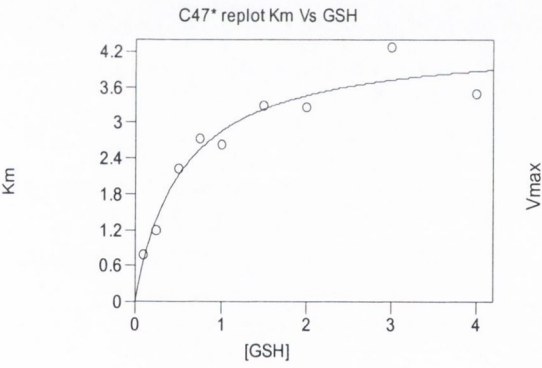
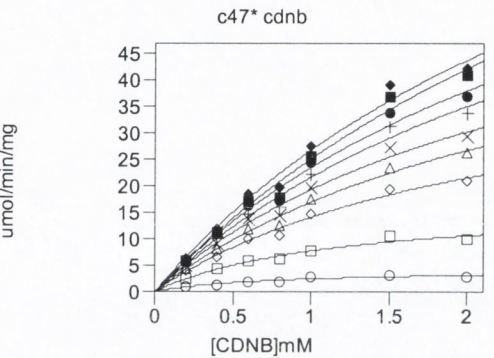
Varying GSH at constant CDNB

| [CDNB] mM | Km    |        | Vmax   |        |
|-----------|-------|--------|--------|--------|
| 0.2       | 0.443 | ±0.037 | 7.043  | ±0.163 |
| 0.4       | 0.552 | ±0.047 | 13.745 | ±0.355 |
| 0.6       | 0.595 | ±0.056 | 21.105 | ±0.623 |
| 0.8       | 0.600 | ±0.063 | 22.345 | ±0.727 |
| 1         | 0.669 | ±0.066 | 32.231 | ±1.031 |
| 1.5       | 0.760 | ±0.064 | 46.854 | ±1.355 |
| 2         | 0.813 | ±0.095 | 51.977 | ±2.122 |



Varying CDNB at constant GSH

| [GSH] mM | Km    |        | Vmax    |        |
|----------|-------|--------|---------|--------|
| 0.1      | 0.784 | ±0.285 | 4.405   | ±0.696 |
| 0.25     | 1.194 | ±0.319 | 17.008  | ±2.339 |
| 0.5      | 2.218 | ±0.561 | 45.555  | ±7.336 |
| 0.75     | 2.740 | ±0.703 | 63.547  | ±11.02 |
| 1        | 2.618 | ±0.708 | 70.077  | ±12.66 |
| 1.5      | 3.280 | ±1.099 | 92.826  | ±22.06 |
| 2        | 3.273 | ±1.026 | 100.526 | ±22.35 |
| 3        | 4.281 | ±1.698 | 132.894 | ±39.83 |
| 4        | 3.500 | ±1.211 | 120.719 | ±30.09 |

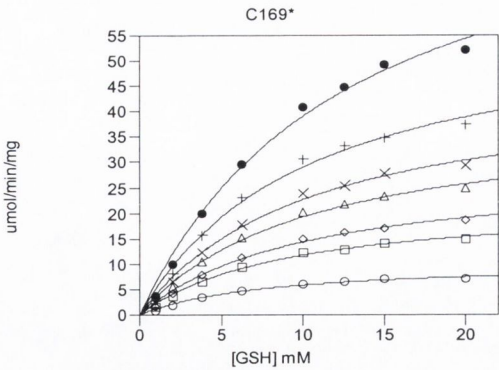


|      | GSH    |        | CDNB   |        |
|------|--------|--------|--------|--------|
| Vmax | 181.79 | ±17.29 | 188.33 | ±64.32 |
| Km   | 0.85   | ±0.048 | 4.39   | ±0.317 |

C169\*

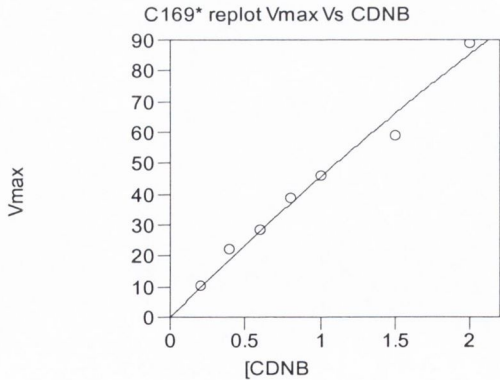
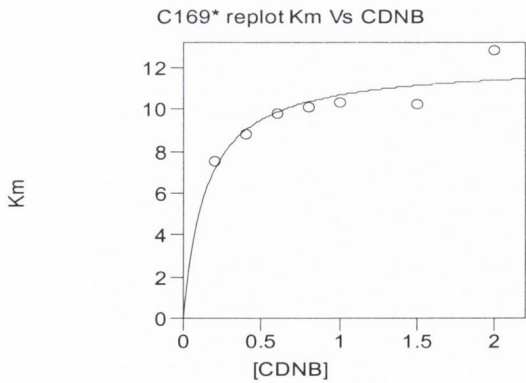
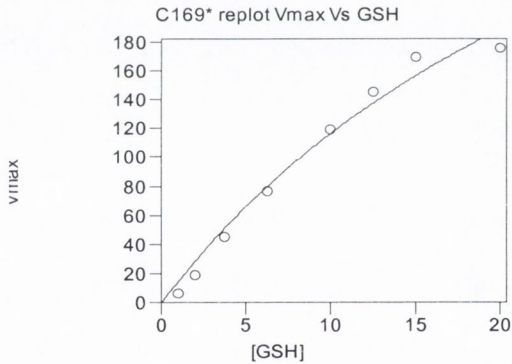
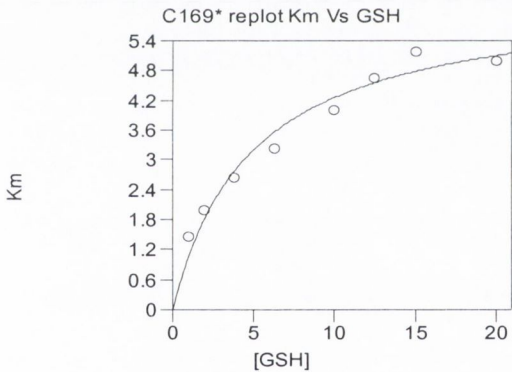
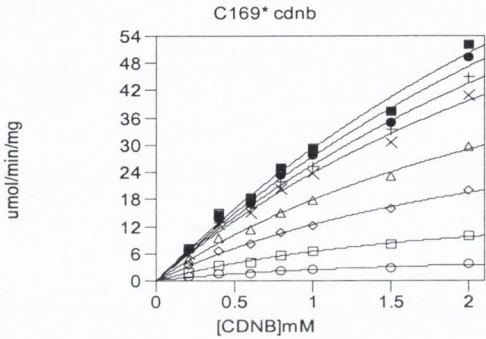
Varying GSH at constant CDNB

| [CDNB] mM | Km            | Vmax          |
|-----------|---------------|---------------|
| 0.2       | 7.544 ±0.997  | 10.286 ±0.563 |
| 0.4       | 8.835 ±1.276  | 22.169 ±1.417 |
| 0.6       | 9.771 ±1.410  | 28.412 ±1.892 |
| 0.8       | 10.080 ±1.435 | 38.683 ±2.575 |
| 1         | 10.312 ±1.607 | 46.045 ±3.386 |
| 1.5       | 10.208 ±1.755 | 58.737 ±4.745 |
| 2         | 12.792 ±2.252 | 89.093 ±8.065 |



Varying CDNB at constant GSH

| [GSH] mM | Km           | Vmax           |
|----------|--------------|----------------|
| 1        | 1.456 ±0.313 | 6.022 ±0.717   |
| 2        | 1.978 ±0.302 | 19.216 ±1.801  |
| 3.75     | 2.643 ±0.449 | 45.184 ±5.147  |
| 6.25     | 3.236 ±0.651 | 75.995 ±10.80  |
| 10       | 3.995 ±1.077 | 119.097 ±23.89 |
| 12.5     | 4.664 ±1.283 | 145.177 ±30.72 |
| 15       | 5.181 ±2.144 | 169.843 ±55.23 |
| 20       | 5.002 ±1.773 | 175.779 ±48.62 |

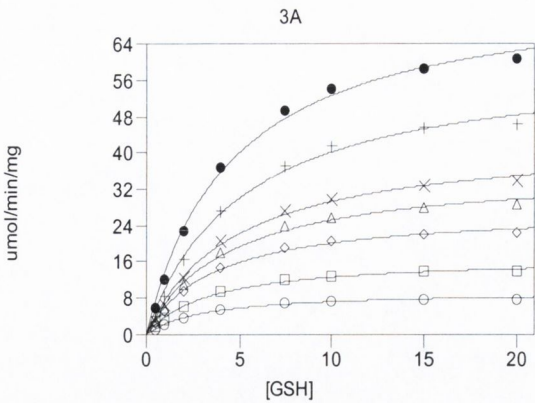


|      | GSH            | CDNB           |
|------|----------------|----------------|
| Vmax | 494.59 ±145.69 | 693.26 ±666.85 |
| Km   | 6.36 ±0.459    | 12.18 ±0.723   |

3A

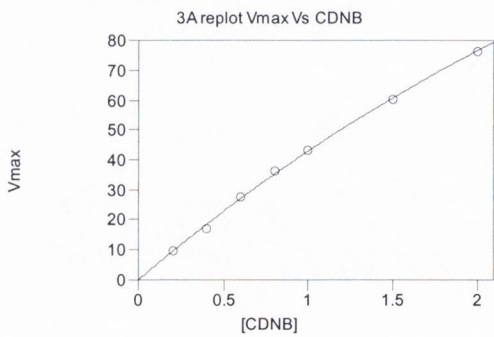
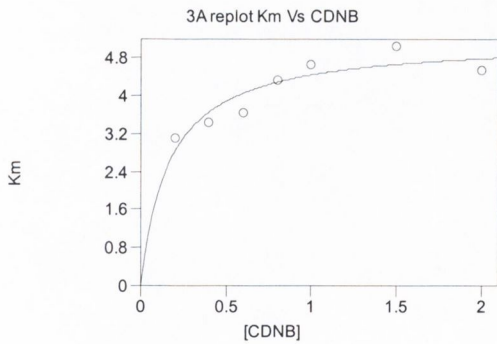
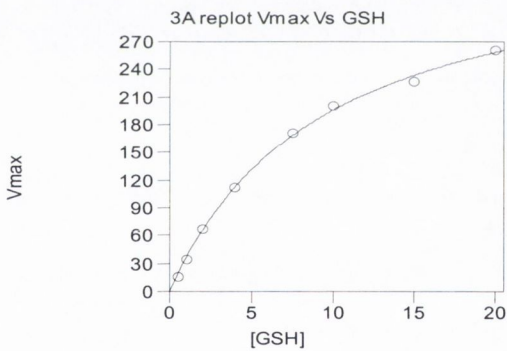
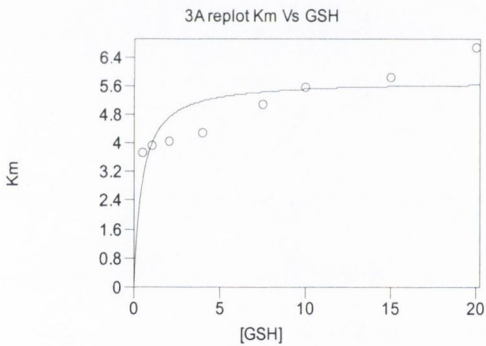
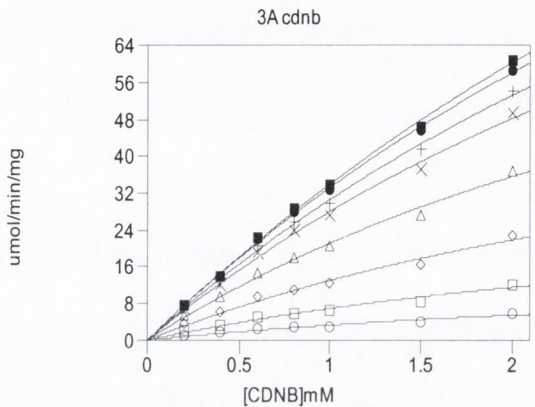
Varying GSH at constant CDNB

| [CDNB] mM | Km           | Vmax          |
|-----------|--------------|---------------|
| 0.2       | 3.103 ±0.319 | 9.499 ±0.293  |
| 0.4       | 3.426 ±0.351 | 16.949 ±0.539 |
| 0.6       | 3.639 ±0.403 | 27.467 ±0.965 |
| 0.8       | 4.338 ±0.425 | 36.191 ±1.202 |
| 1         | 4.668 ±0.459 | 42.995 ±1.472 |
| 1.5       | 5.038 ±0.510 | 60.457 ±2.193 |
| 2         | 4.535 ±0.375 | 76.524 ±2.181 |



Varying CDNB at constant GSH

| [GSH] mM | Km           | Vmax           |
|----------|--------------|----------------|
| 0.5      | 3.744 ±2.088 | 15.892 ±6.494  |
| 1        | 3.928 ±1.822 | 34.391 ±11.81  |
| 2        | 4.059 ±1.379 | 66.164 ±16.78  |
| 4        | 4.282 ±1.178 | 111.768 ±23.23 |
| 7.5      | 5.066 ±1.183 | 170.018 ±31.05 |
| 10       | 5.543 ±1.070 | 200.411 ±30.79 |
| 15       | 5.837 ±0.809 | 227.242 ±25.31 |
| 20       | 6.663 ±1.281 | 260.556 ±41.18 |

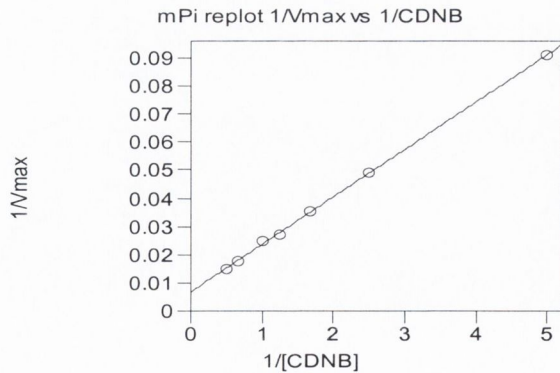
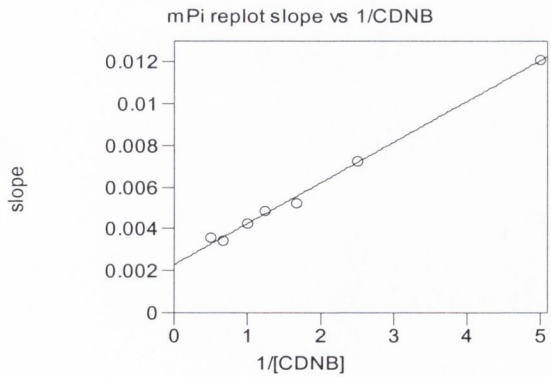
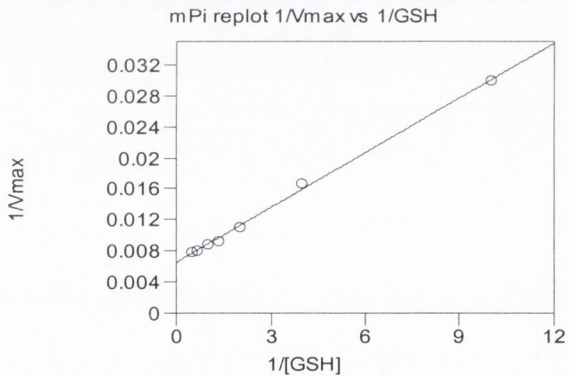
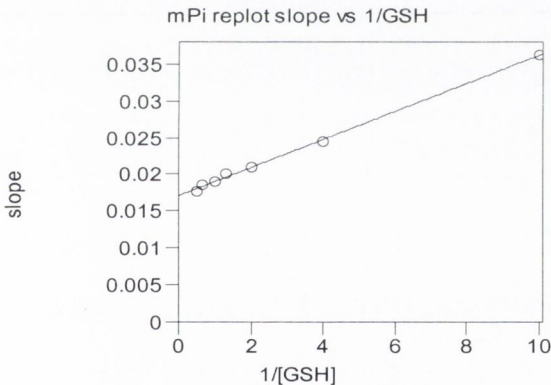
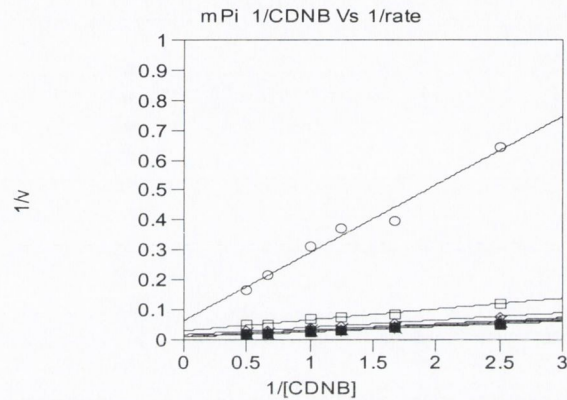
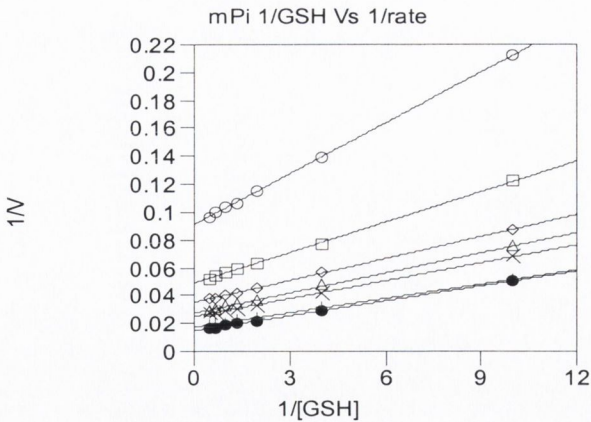


|      |               |               |
|------|---------------|---------------|
| Vmax | GSH           | CDNB          |
| Km   | 381.84 ±11.21 | 351.02 ±41.36 |
|      | 5.18 ±0.31    | 5.74 ±0.38    |



| [CDNB] | 1/Vmax   | Vmax     | Km/Vmax  |
|--------|----------|----------|----------|
| 0.2    | 9.11E-02 | 1.10E+01 | 1.21E-02 |
| 0.4    | 4.91E-02 | 2.04E+01 | 7.28E-03 |
| 0.6    | 3.51E-02 | 2.85E+01 | 5.25E-03 |
| 0.8    | 2.73E-02 | 3.66E+01 | 4.82E-03 |
| 1      | 2.50E-02 | 4.00E+01 | 4.28E-03 |
| 1.5    | 1.75E-02 | 5.72E+01 | 3.41E-03 |
| 2      | 1.47E-02 | 6.78E+01 | 3.57E-03 |

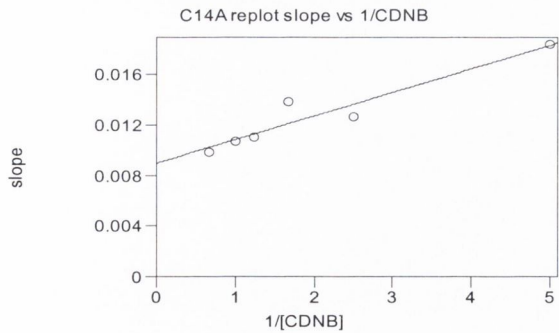
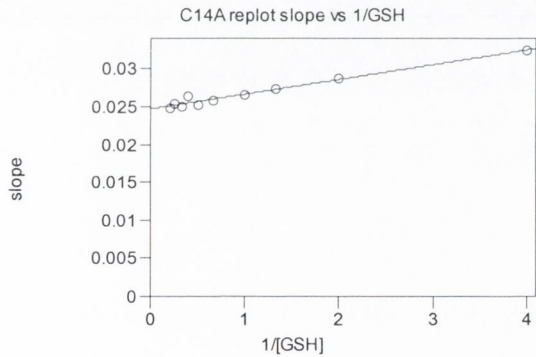
| [GSH] | 1/Vmax   | Vmax     | Km/Vmax  |
|-------|----------|----------|----------|
| 0.025 | 6.37E-02 | 1.57E+01 | 2.27E-01 |
| 0.1   | 2.99E-02 | 3.34E+01 | 3.63E-02 |
| 0.25  | 1.66E-02 | 6.04E+01 | 2.44E-02 |
| 0.5   | 1.11E-02 | 9.00E+01 | 2.09E-02 |
| 0.75  | 9.25E-03 | 1.08E+02 | 2.00E-02 |
| 1     | 8.90E-03 | 1.12E+02 | 1.91E-02 |
| 1.5   | 7.98E-03 | 1.25E+02 | 1.85E-02 |
| 2     | 7.83E-03 | 1.28E+02 | 1.77E-02 |



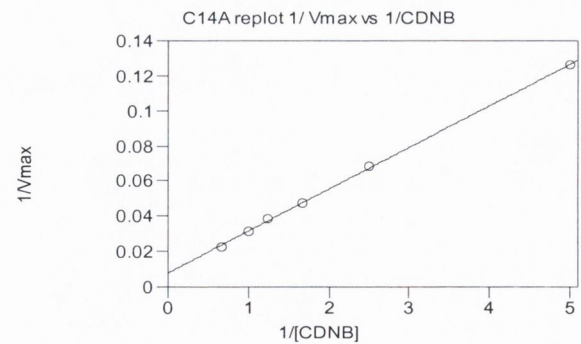
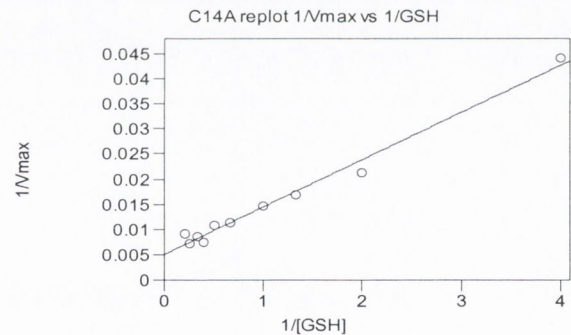
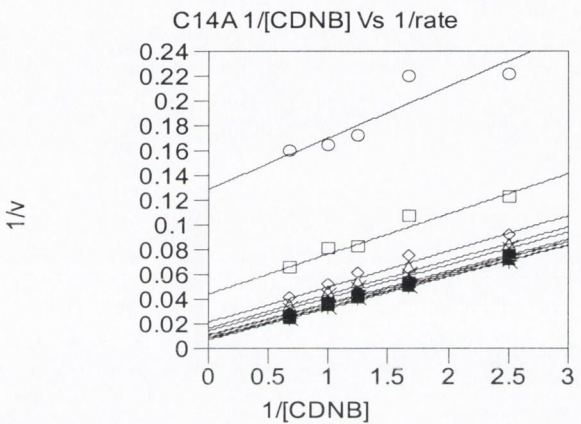
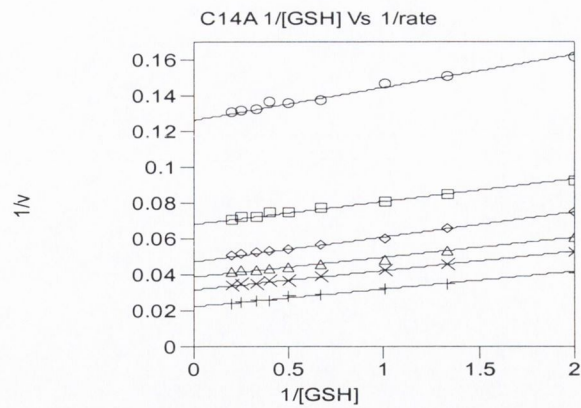
|      | 1/Vmax   | Vmax     | Km/Vmax  | Km       |
|------|----------|----------|----------|----------|
| GSH  | 6.73E-03 | 1.49E+02 | 2.31E-03 | 3.43E-01 |
| CDNB | 6.51E-03 | 1.54E+02 | 1.71E-02 | 2.62E+00 |

| [CDNB] | 1/Vmax   | Vmax     | Km/Vmax  |
|--------|----------|----------|----------|
| 0.2    | 1.26E-01 | 7.93E+00 | 1.84E-02 |
| 0.4    | 6.80E-02 | 1.47E+01 | 1.27E-02 |
| 0.6    | 4.71E-02 | 2.12E+01 | 1.39E-02 |
| 0.8    | 3.88E-02 | 2.58E+01 | 1.10E-02 |
| 1      | 3.16E-02 | 3.17E+01 | 1.07E-02 |
| 1.5    | 2.22E-02 | 4.50E+01 | 9.78E-03 |

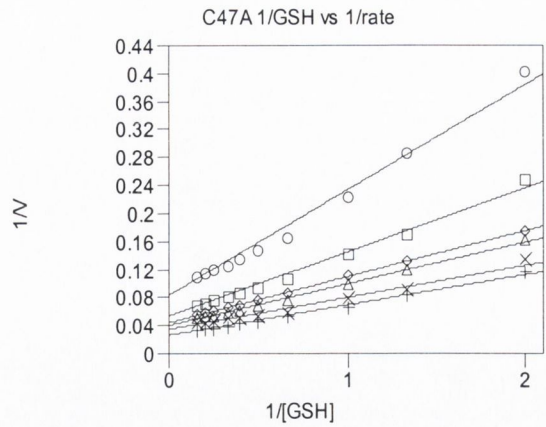
| [GSH] | 1/Vmax   | Vmax     | Km/Vmax  |
|-------|----------|----------|----------|
| 0.1   | 1.29E-01 | 7.75E+00 | 4.12E-02 |
| 0.25  | 4.41E-02 | 2.27E+01 | 3.25E-02 |
| 0.5   | 2.13E-02 | 4.69E+01 | 2.87E-02 |
| 0.75  | 1.67E-02 | 6.01E+01 | 2.73E-02 |
| 1     | 1.45E-02 | 6.91E+01 | 2.66E-02 |
| 1.5   | 1.12E-02 | 8.89E+01 | 2.58E-02 |
| 2     | 1.08E-02 | 9.28E+01 | 2.53E-02 |
| 2.5   | 7.49E-03 | 1.33E+02 | 2.64E-02 |
| 3     | 8.56E-03 | 1.17E+02 | 2.51E-02 |
| 4     | 7.19E-03 | 1.39E+02 | 2.55E-02 |
| 5     | 9.03E-03 | 1.11E+02 | 2.49E-02 |



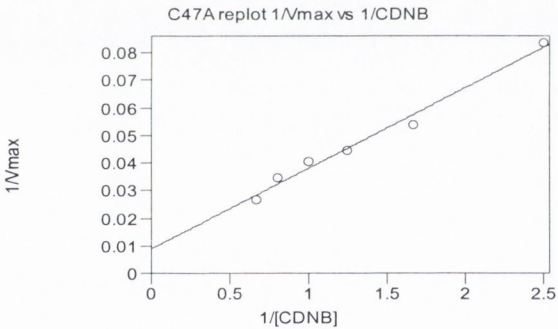
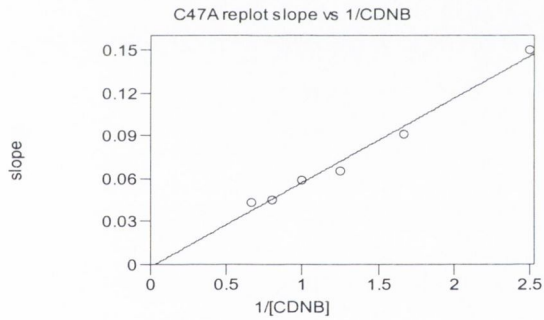
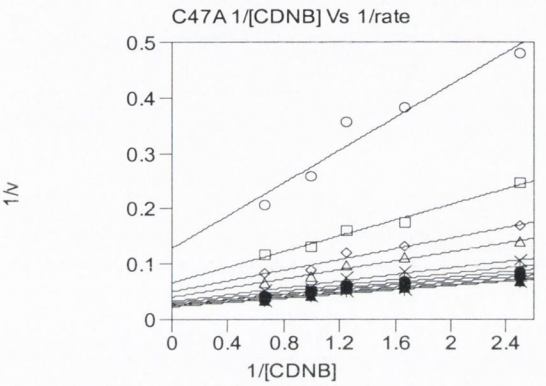
|      | 1/Vmax   | Vmax     | Km/Vmax  | Km       |
|------|----------|----------|----------|----------|
| GSH  | 7.83E-03 | 1.28E+02 | 8.96E-03 | 1.14E+00 |
| CDNB | 5.04E-03 | 1.98E+02 | 2.47E-02 | 4.91E+00 |



| [CDNB] | 1/Vmax   | Vmax     | Km/Vmax  |
|--------|----------|----------|----------|
| 0.4    | 8.35E-02 | 1.20E+01 | 1.50E-01 |
| 0.6    | 5.40E-02 | 1.85E+01 | 9.09E-02 |
| 0.8    | 4.43E-02 | 2.26E+01 | 6.53E-02 |
| 1      | 4.03E-02 | 2.48E+01 | 5.91E-02 |
| 1.25   | 3.47E-02 | 2.88E+01 | 4.53E-02 |
| 1.5    | 2.65E-02 | 3.78E+01 | 4.29E-02 |



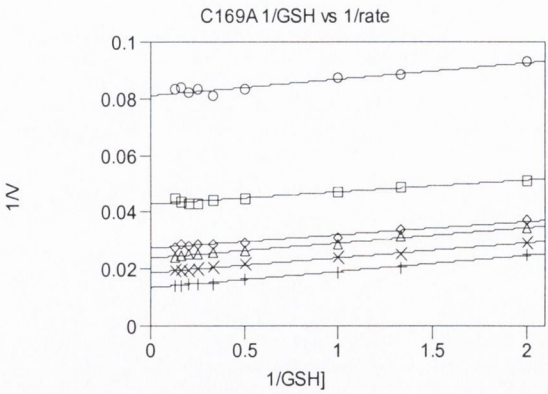
| [GSH] | 1/Vmax   | Vmax     | Km/Vmax  |
|-------|----------|----------|----------|
| 0.25  | 1.29E-01 | 7.76E+00 | 1.47E-01 |
| 0.5   | 6.65E-02 | 1.50E+01 | 7.07E-02 |
| 0.75  | 5.06E-02 | 1.98E+01 | 4.81E-02 |
| 1     | 4.08E-02 | 2.45E+01 | 4.07E-02 |
| 1.5   | 3.32E-02 | 3.01E+01 | 2.99E-02 |
| 2     | 3.01E-02 | 3.33E+01 | 2.64E-02 |
| 2.5   | 2.69E-02 | 3.72E+01 | 2.47E-02 |
| 3     | 2.74E-02 | 3.65E+01 | 2.20E-02 |
| 4     | 2.45E-02 | 4.09E+01 | 2.13E-02 |
| 5     | 2.55E-02 | 3.92E+01 | 1.88E-02 |
| 6.25  | 2.33E-02 | 4.29E+01 | 1.89E-02 |



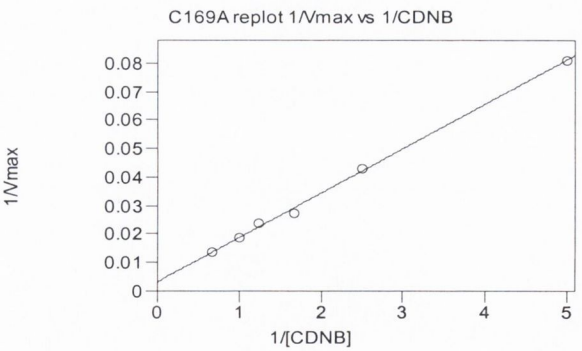
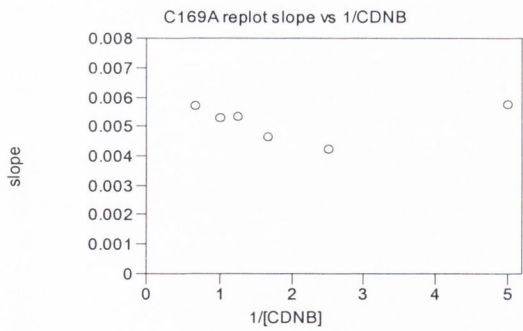
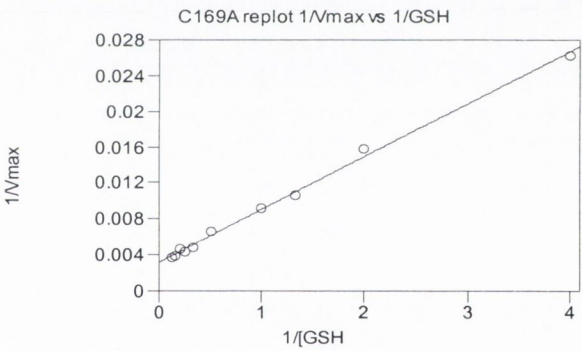
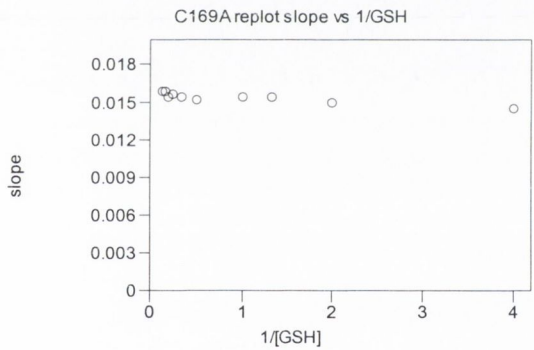
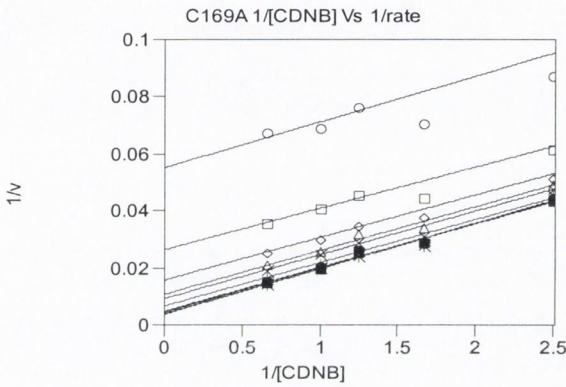
|      | 1/Vmax   | Vmax     | Km/Vmax   | Km |
|------|----------|----------|-----------|----|
| GSH  |          |          |           |    |
| CDNB | 8.87E-03 | 1.13E+02 | -1.66E-03 |    |



| [CDNB] | 1/Vmax   | Vmax     | Km/Vmax  |
|--------|----------|----------|----------|
| 0.2    | 8.11E-02 | 1.23E+01 | 5.75E-03 |
| 0.4    | 4.29E-02 | 2.33E+01 | 4.21E-03 |
| 0.6    | 2.74E-02 | 3.65E+01 | 4.65E-03 |
| 0.8    | 2.39E-02 | 4.18E+01 | 5.32E-03 |
| 1      | 1.87E-02 | 5.34E+01 | 5.30E-03 |
| 1.5    | 1.35E-02 | 7.43E+01 | 5.70E-03 |



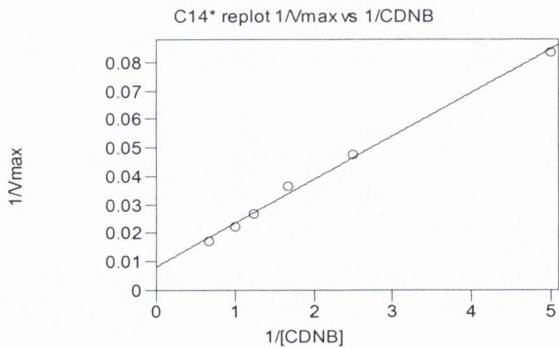
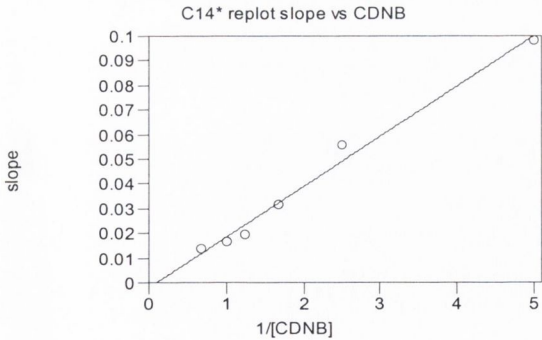
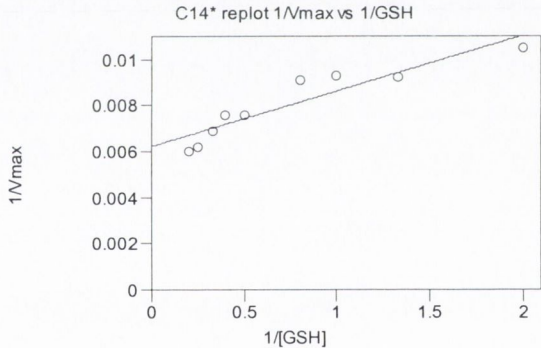
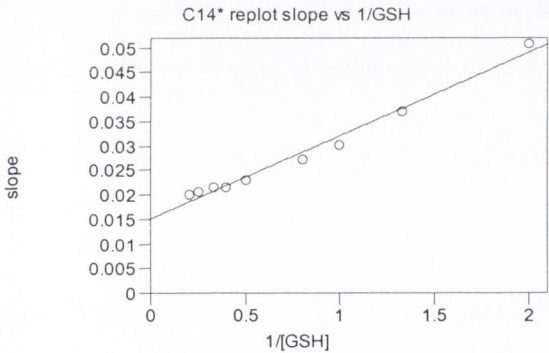
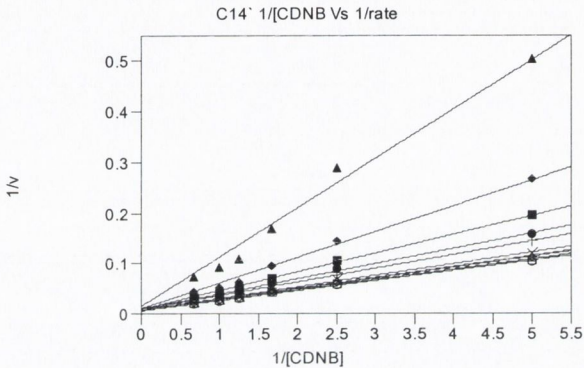
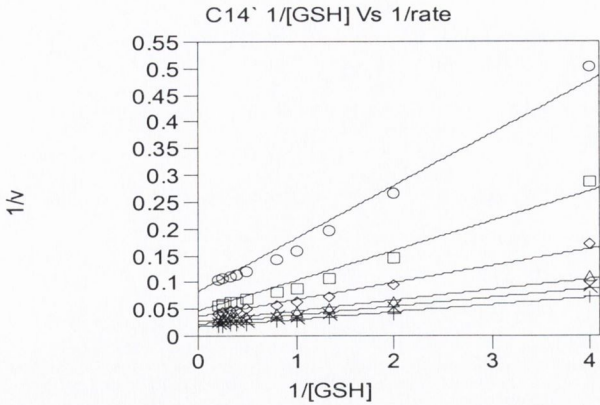
| [GSH] | 1/Vmax   | Vmax     | Km/Vmax  |
|-------|----------|----------|----------|
| 0.1   | 5.52E-02 | 1.81E+01 | 1.60E-02 |
| 0.25  | 2.63E-02 | 3.80E+01 | 1.45E-02 |
| 0.5   | 1.57E-02 | 6.38E+01 | 1.50E-02 |
| 0.75  | 1.07E-02 | 9.36E+01 | 1.54E-02 |
| 1     | 9.19E-03 | 1.09E+02 | 1.54E-02 |
| 2     | 6.61E-03 | 1.51E+02 | 1.52E-02 |
| 3     | 4.78E-03 | 2.09E+02 | 1.54E-02 |
| 4     | 4.41E-03 | 2.27E+02 | 1.56E-02 |
| 5     | 4.65E-03 | 2.15E+02 | 1.54E-02 |
| 6.25  | 3.91E-03 | 2.56E+02 | 1.59E-02 |
| 7.5   | 3.64E-03 | 2.75E+02 | 1.59E-02 |



|      | 1/Vmax   | Vmax     |
|------|----------|----------|
| GSH  | 3.17E-03 | 3.15E+02 |
| CDNB | 3.17E-03 | 3.16E+02 |

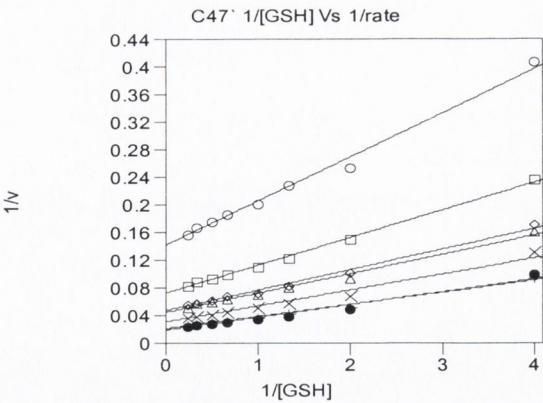
| [CDNB] | 1/Vmax   | Vmax     | Km/Vmax  |
|--------|----------|----------|----------|
| 0.2    | 8.36E-02 | 1.20E+01 | 9.81E-02 |
| 0.4    | 4.77E-02 | 2.10E+01 | 5.55E-02 |
| 0.6    | 3.64E-02 | 2.74E+01 | 3.14E-02 |
| 0.8    | 2.69E-02 | 3.72E+01 | 1.98E-02 |
| 1      | 2.21E-02 | 4.52E+01 | 1.64E-02 |
| 1.5    | 1.73E-02 | 5.79E+01 | 1.36E-02 |

| [GSH] | 1/Vmax   | Vmax     | Km/Vmax  |
|-------|----------|----------|----------|
| 0.25  | 1.51E-02 | 6.61E+01 | 9.75E-02 |
| 0.5   | 1.05E-02 | 9.54E+01 | 5.09E-02 |
| 0.75  | 9.24E-03 | 1.08E+02 | 3.71E-02 |
| 1     | 9.31E-03 | 1.07E+02 | 3.01E-02 |
| 1.25  | 9.08E-03 | 1.10E+02 | 2.71E-02 |
| 2     | 7.57E-03 | 1.32E+02 | 2.30E-02 |
| 2.5   | 7.57E-03 | 1.32E+02 | 2.15E-02 |
| 3     | 6.92E-03 | 1.44E+02 | 2.14E-02 |
| 4     | 6.22E-03 | 1.61E+02 | 2.05E-02 |
| 5     | 6.02E-03 | 1.66E+02 | 2.00E-02 |

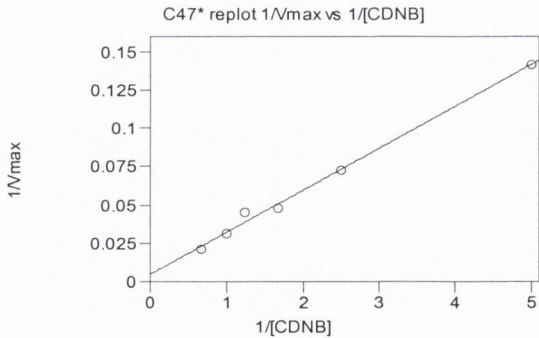
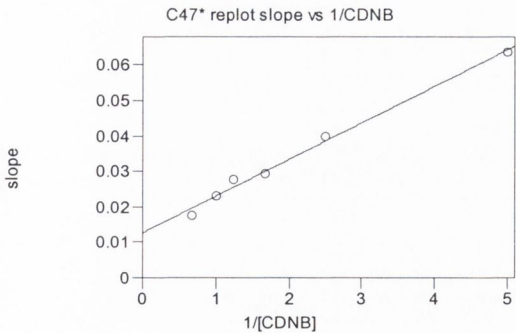
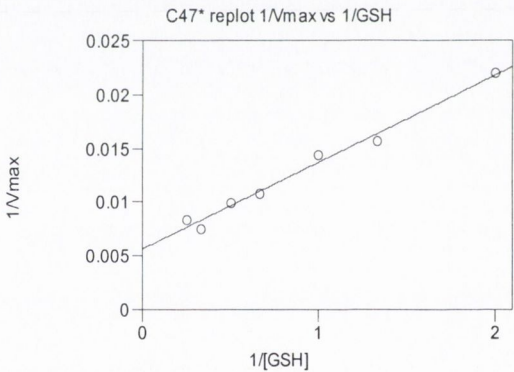
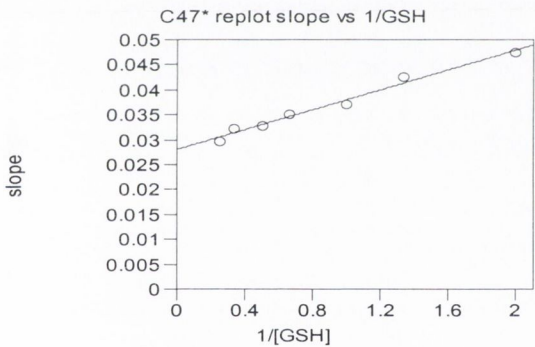
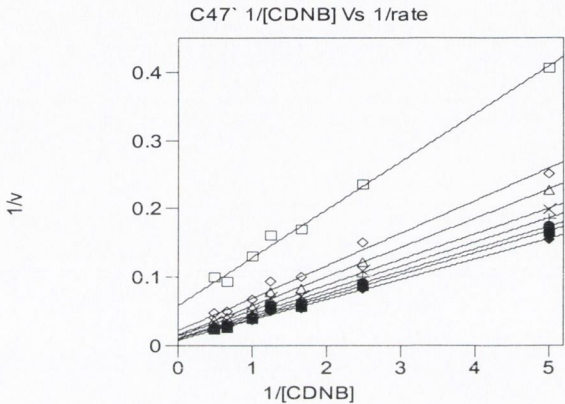


|      | 1/Vmax   | Vmax     | Km/Vmax   |
|------|----------|----------|-----------|
| GSH  | 6.23E-03 | 1.61E+02 | 1.51E-02  |
| CDNB | 8.25E-03 | 1.21E+02 | -1.93E-03 |

| [CDNB] | 1/Vmax   | Vmax     | Km/Vmax  |
|--------|----------|----------|----------|
| 0.2    | 1.42E-01 | 7.04E+00 | 6.36E-02 |
| 0.4    | 7.28E-02 | 1.37E+01 | 3.98E-02 |
| 0.6    | 4.74E-02 | 2.11E+01 | 2.94E-02 |
| 0.8    | 4.48E-02 | 2.23E+01 | 2.78E-02 |
| 1      | 3.10E-02 | 3.22E+01 | 2.29E-02 |
| 1.5    | 2.13E-02 | 4.69E+01 | 1.74E-02 |



| [GSH] | 1/Vmax   | Vmax     | Km/Vmax  |
|-------|----------|----------|----------|
| 0.25  | 5.88E-02 | 1.70E+01 | 6.99E-02 |
| 0.5   | 2.20E-02 | 4.56E+01 | 4.74E-02 |
| 0.75  | 1.57E-02 | 6.35E+01 | 4.26E-02 |
| 1     | 1.43E-02 | 7.01E+01 | 3.72E-02 |
| 1.5   | 1.08E-02 | 9.28E+01 | 3.52E-02 |
| 2     | 9.95E-03 | 1.01E+02 | 3.29E-02 |
| 3     | 7.53E-03 | 1.33E+02 | 3.21E-02 |
| 4     | 8.28E-03 | 1.21E+02 | 2.96E-02 |

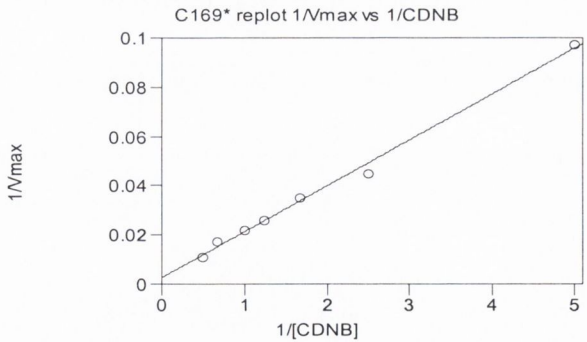
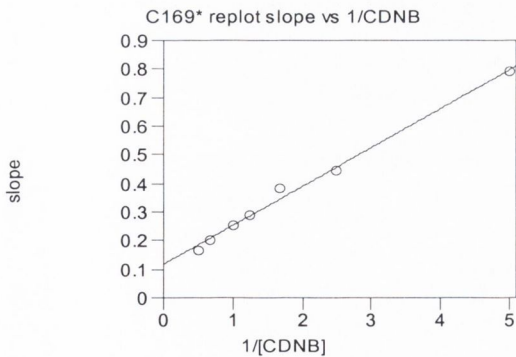
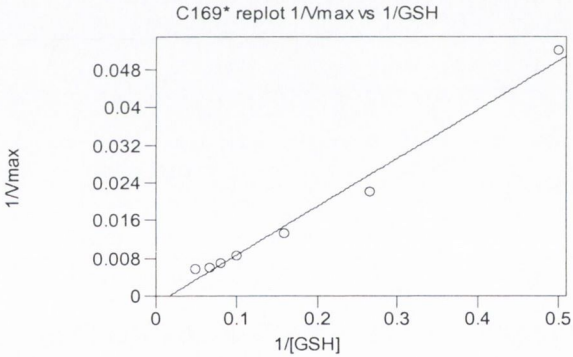
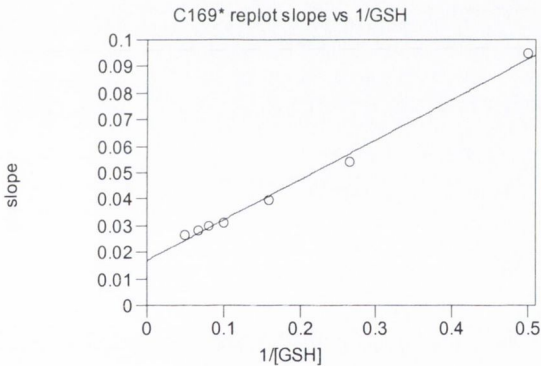
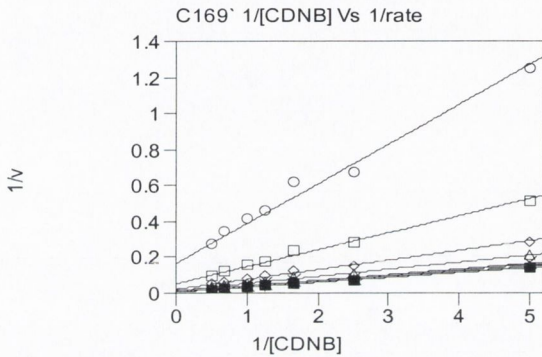
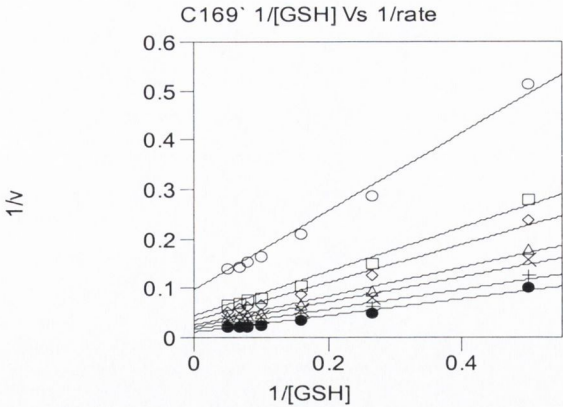


|      | 1/Vmax   | Vmax     | Km/Vmax  | Km       |
|------|----------|----------|----------|----------|
| GSH  | 4.60E-03 | 2.17E+02 | 1.27E-02 | 2.76E+00 |
| CDNB | 5.64E-03 | 1.77E+02 | 2.81E-02 | 4.97E+00 |



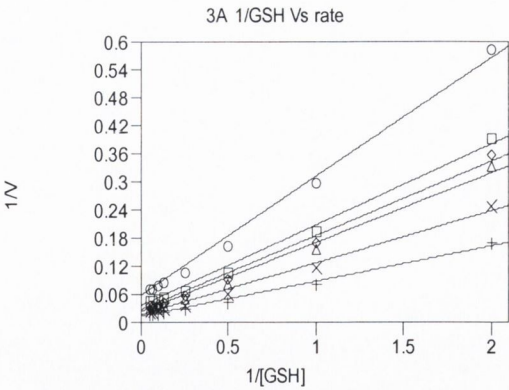
| [CDNB] | 1/Vmax   | Vmax     | Km/Vmax  |
|--------|----------|----------|----------|
| 0.2    | 9.72E-02 | 1.03E+01 | 7.92E-01 |
| 0.4    | 4.51E-02 | 2.22E+01 | 4.44E-01 |
| 0.6    | 3.52E-02 | 2.84E+01 | 3.83E-01 |
| 0.8    | 2.59E-02 | 3.87E+01 | 2.91E-01 |
| 1      | 2.17E-02 | 4.60E+01 | 2.53E-01 |
| 1.5    | 1.70E-02 | 5.87E+01 | 2.01E-01 |
| 2      | 1.12E-02 | 8.91E+01 | 1.68E-01 |

| [GSH] | 1/Vmax   | Vmax     | Km/Vmax  |
|-------|----------|----------|----------|
| 1     | 1.66E-01 | 6.02E+00 | 2.21E-01 |
| 2     | 5.20E-02 | 1.92E+01 | 9.47E-02 |
| 3.75  | 2.21E-02 | 4.52E+01 | 5.38E-02 |
| 6.25  | 1.32E-02 | 7.60E+01 | 3.96E-02 |
| 10    | 8.40E-03 | 1.19E+02 | 3.12E-02 |
| 12.5  | 6.89E-03 | 1.45E+02 | 2.99E-02 |
| 15    | 5.89E-03 | 1.70E+02 | 2.79E-02 |
| 20    | 5.69E-03 | 1.76E+02 | 2.66E-02 |

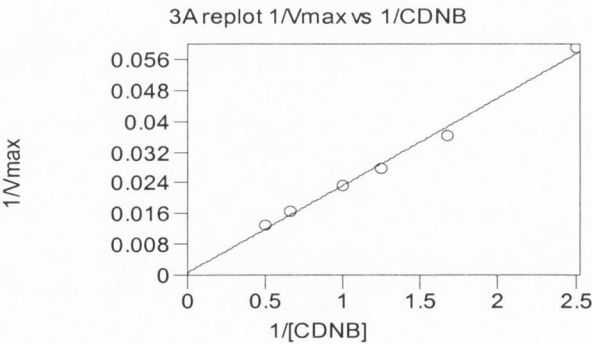
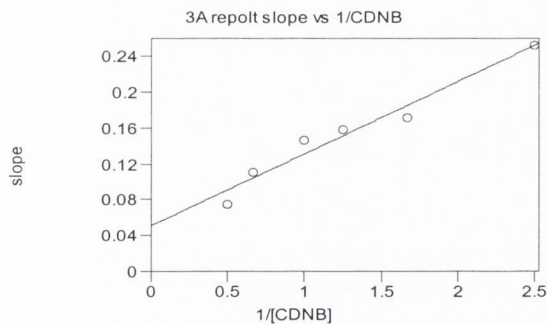
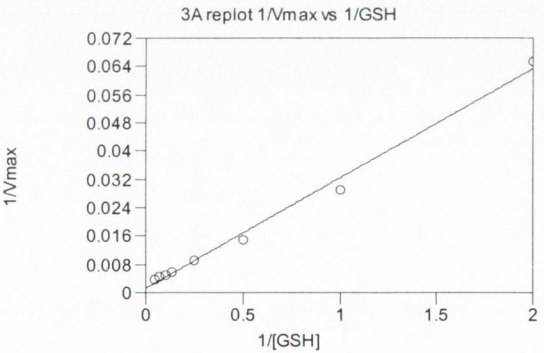
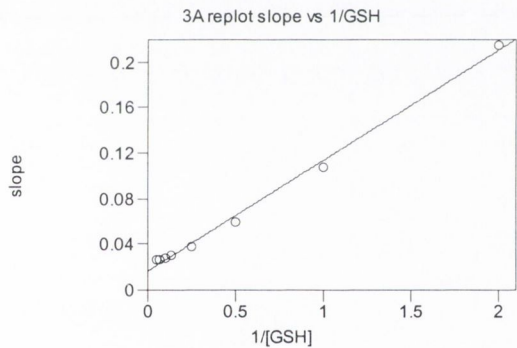
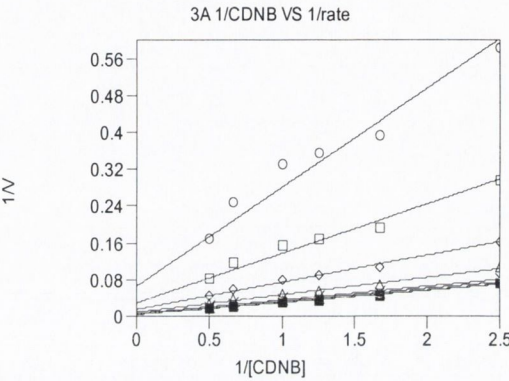


|      | 1/Vmax    | Vmax     | Km/Vmax  | Km       |
|------|-----------|----------|----------|----------|
| GSH  | 2.71E-03  | 3.69E+02 | 1.18E-01 | 1.00E+00 |
| CDNB | -1.72E-03 |          | 1.69E-02 |          |

| [CDNB] | 1/Vmax   | Vmax     | Km/Vmax  |
|--------|----------|----------|----------|
| 0.4    | 5.90E-02 | 1.69E+01 | 2.53E-01 |
| 0.6    | 3.64E-02 | 2.75E+01 | 1.72E-01 |
| 0.8    | 2.76E-02 | 3.62E+01 | 1.58E-01 |
| 1      | 2.33E-02 | 4.30E+01 | 1.47E-01 |
| 1.5    | 1.65E-02 | 6.05E+01 | 1.11E-01 |
| 2      | 1.31E-02 | 7.65E+01 | 7.50E-02 |

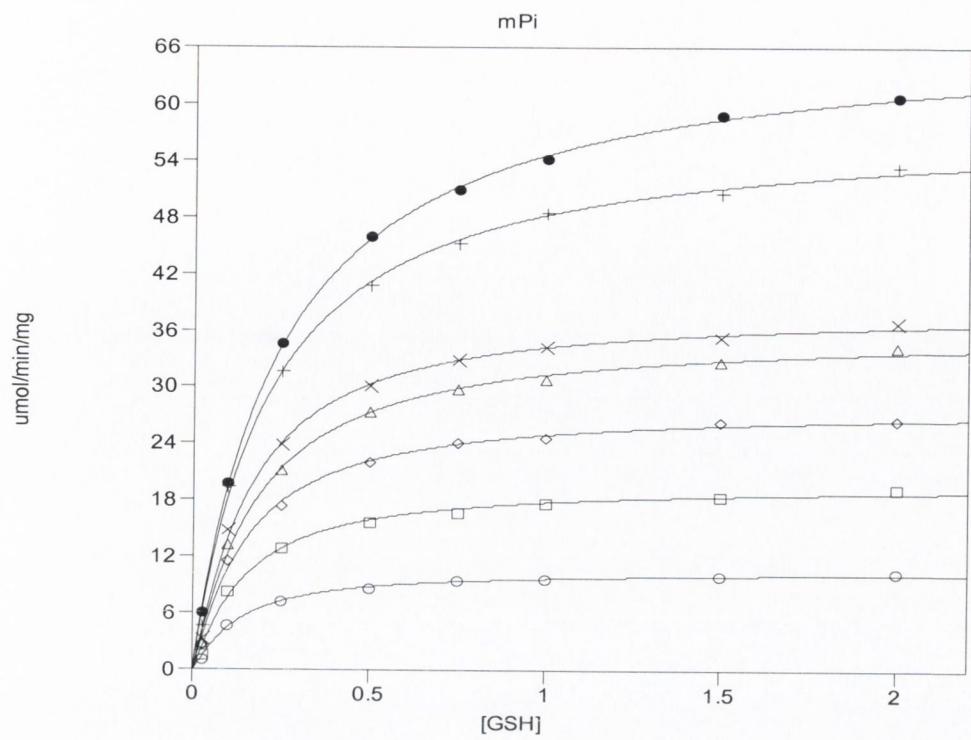


| [GSH] | 1/Vmax   | Vmax     | Km/Vmax  |
|-------|----------|----------|----------|
| 0.5   | 6.53E-02 | 1.53E+01 | 2.15E-01 |
| 1     | 2.91E-02 | 3.44E+01 | 1.08E-01 |
| 2     | 1.51E-02 | 6.62E+01 | 5.92E-02 |
| 4     | 8.95E-03 | 1.12E+02 | 3.79E-02 |
| 7.5   | 5.88E-03 | 1.70E+02 | 2.98E-02 |
| 10    | 4.99E-03 | 2.00E+02 | 2.79E-02 |
| 15    | 4.40E-03 | 2.27E+02 | 2.62E-02 |
| 20    | 3.84E-03 | 2.61E+02 | 2.62E-02 |



|      | 1/Vmax   | Vmax     | Km/Vmax  | Km       |
|------|----------|----------|----------|----------|
| GSH  | 1.29E-03 | 7.77E+02 | 1.65E-02 | 1.28E+01 |
| CDNB | 6.72E-04 | 1.49E+03 | 5.09E-02 | 7.58E+01 |

# Hill Plot



## .2mM CDNB

Km 8.59E-02  
Vmax 1.05E+01  
R<sup>2</sup> 0.997  
n 1.20E+00

## .4mM CDNB

Km 1.04E-01  
Vmax 1.96E+01  
R<sup>2</sup> 0.995  
n 1.17E+00

## .6mM CDNB

Km 1.16E-01  
Vmax 2.78E+01  
R<sup>2</sup> 0.997  
n 1.14E+00

## .8mM CDNB

Km 1.44E-01  
Vmax 3.58E+01  
R<sup>2</sup> 0.997  
n 1.12E+00

## 1mM CDNB

Km 1.15E-01  
Vmax 3.81E+01  
R<sup>2</sup> 9.98E-01  
n 1.17E+00

## 1.5mM CDNB

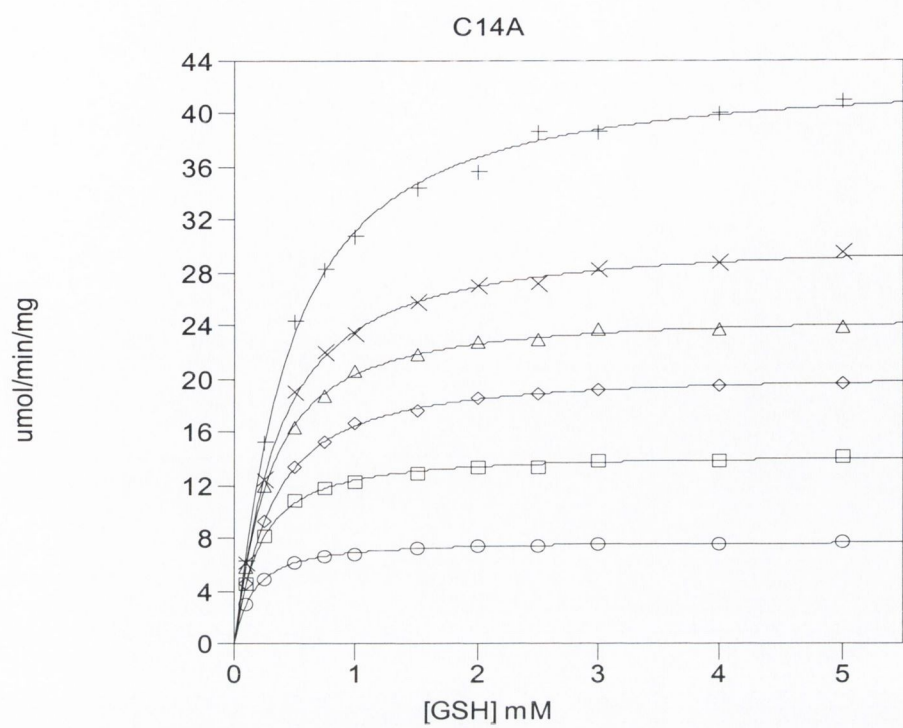
Km 1.98E-01  
Vmax 5.78E+01  
R<sup>2</sup> 9.98E-01  
n 1.03E+00

## 2mM CDNB

Km 2.45E-01  
Vmax 6.80E+01  
R<sup>2</sup> 0.999  
n 9.97E-01



Hill Plot



.2mM CDNB

Km 1.25E-01  
Vmax 7.78E+00  
R<sup>2</sup> 0.998  
n 1.11E+00

.4mM CDNB

Km 1.56E-01  
Vmax 1.43E+01  
R<sup>2</sup> 0.998  
n 1.14E+00

.6mM CDNB

Km 2.37E-01  
Vmax 2.04E+01  
R<sup>2</sup> 0.999  
n 1.17E+00

.8mM CDNB

Km 2.24E-01  
Vmax 2.49E+01  
R<sup>2</sup> 0.998  
n 1.16E+00

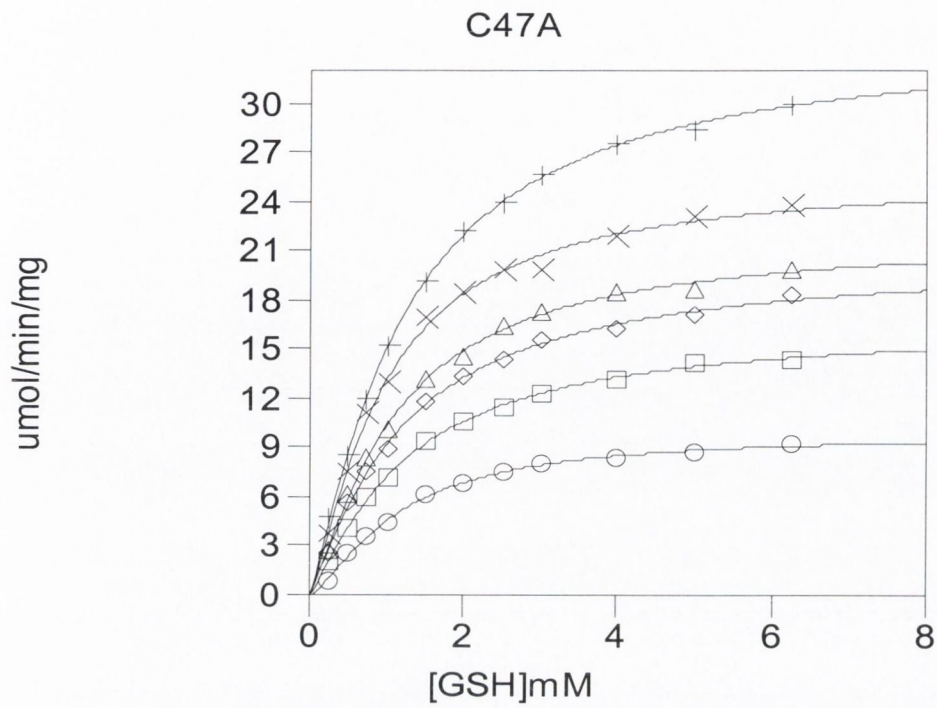
1mM CDNB

Km 2.90E-01  
Vmax 3.05E+01  
R<sup>2</sup> 9.98E-01  
n 1.14E+00

1.5mM CDNB

Km 3.80E-01  
Vmax 4.30E+01  
R<sup>2</sup> 9.97E-01  
n 1.14E+00

Hill Plot



.4mM CDNB

Km 1.17E+00  
Vmax 9.86E+00  
R<sup>2</sup> 0.998  
n 1.44E+00

.6mM CDNB

Km 1.28E+00  
Vmax 1.65E+01  
R<sup>2</sup> 0.999  
n 1.22E+00

.8mM CDNB

Km 1.24E+00  
Vmax 2.06E+01  
R<sup>2</sup> 0.998  
n 1.17E+00

1mM CDNB

Km 1.12E+00  
Vmax 2.19E+01  
R<sup>2</sup> 0.998  
n 1.26E+00

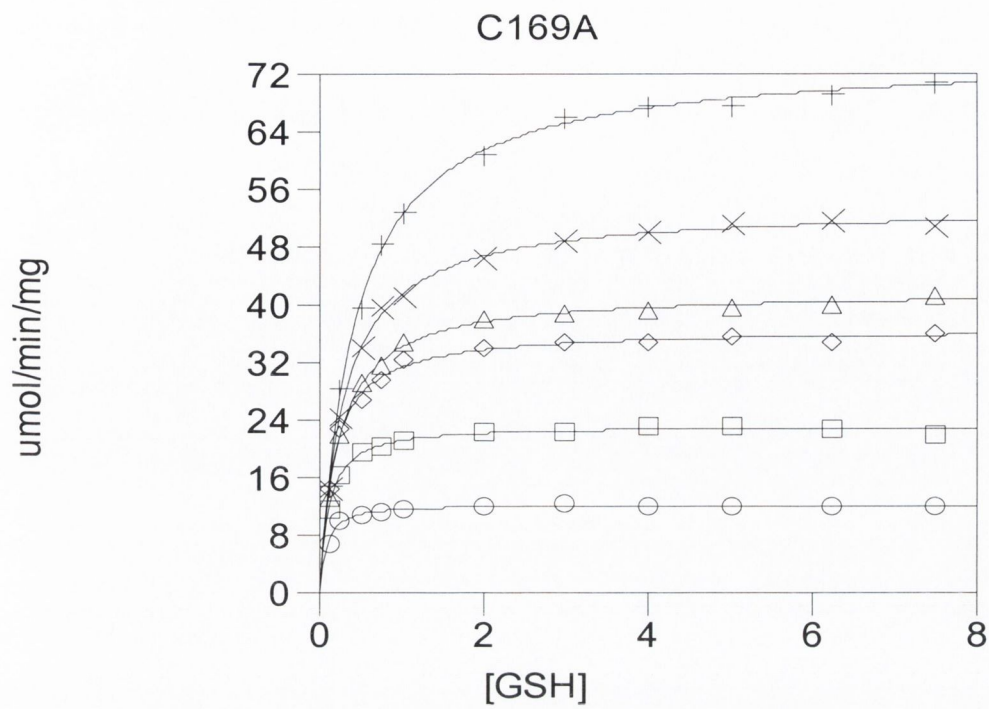
1.25mM CDNB

Km 9.49E-01  
Vmax 2.56E+01  
R<sup>2</sup> 9.96E-01  
n 1.28E+00

1.5mM CDNB

Km 1.25E+00  
Vmax 3.39E+01  
R<sup>2</sup> 9.99E-01  
n 1.21E+00

Hill Plot



.2mM CDNB

Km 5.43E-02  
Vmax 1.22E+01  
R<sup>2</sup> 0.99  
n 1.13E+00

.4mM CDNB

Km 9.55E-02  
Vmax 2.32E+01  
R<sup>2</sup> 0.992  
n 1.04E+00

.6mM CDNB

Km 1.66E-01  
Vmax 3.66E+01  
R<sup>2</sup> 0.994  
n 9.76E-01

.8mM CDNB

Km 2.25E-01  
Vmax 4.19E+01  
R<sup>2</sup> 0.997  
n 9.89E-01

1mM CDNB

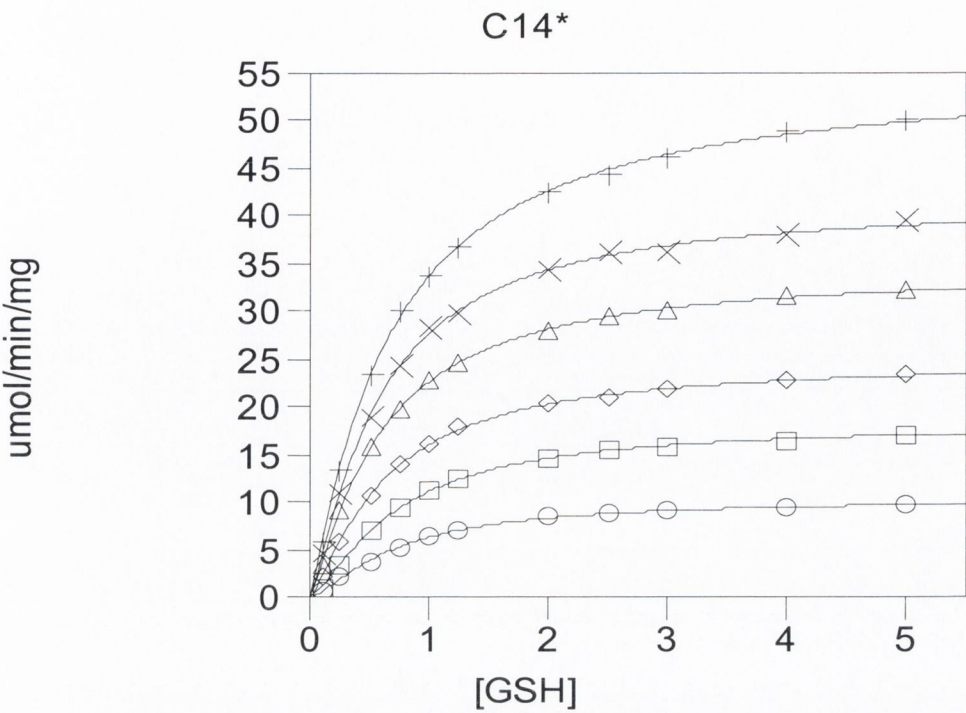
Km 2.92E-01  
Vmax 5.36E+01  
R<sup>2</sup> 9.98E-01  
n 9.80E-01

1.5mM CDNB

Km 4.24E-01  
Vmax 7.46E+01  
R<sup>2</sup> 9.98E-01  
n 9.81E-01



Hill Plot



.2mM CDNB

Km 6.49E-01  
Vmax 1.03E+01  
R<sup>2</sup> 0.999  
n 1.40E+00

.4mM CDNB

Km 6.41E-01  
Vmax 1.82E+01  
R<sup>2</sup> 0.999  
n 1.37E+00

.6mM CDNB

Km 5.47E-01  
Vmax 2.49E+01  
R<sup>2</sup> 0.999  
n 1.28E+00

.8mM CDNB

Km 5.20E-01  
Vmax 3.44E+01  
R<sup>2</sup> 0.999  
n 1.21E+00

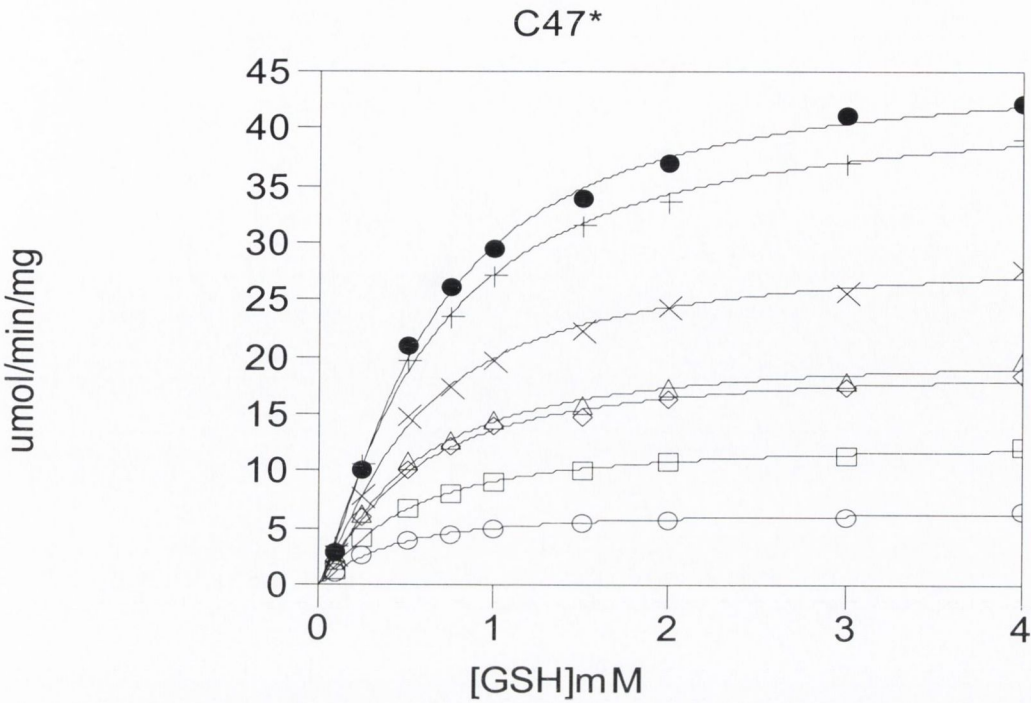
1mM CDNB

Km 5.03E-01  
Vmax 4.17E+01  
R<sup>2</sup> 9.98E-01  
n 1.23E+00

1.5mM CDNB

Km 6.31E-01  
Vmax 5.51E+01  
R<sup>2</sup> 9.99E-01  
n 1.12E+00

Hill Plot



.2mM CDNB

Km 3.24E-01  
Vmax 6.60E+00  
R<sup>2</sup> 0.995  
n 1.20E+00

.4mM CDNB

Km 3.91E-01  
Vmax 1.27E+01  
R<sup>2</sup> 0.997  
n 1.23E+00

.6mM CDNB

Km 4.14E-01  
Vmax 1.93E+01  
R<sup>2</sup> 0.996  
n 1.24E+00

.8mM CDNB

Km 4.19E-01  
Vmax 2.04E+01  
R<sup>2</sup> 0.994  
n 1.24E+00

1mM CDNB

Km 4.57E-01  
Vmax 2.90E+01  
R<sup>2</sup> 9.96E-01  
n 1.27E+00

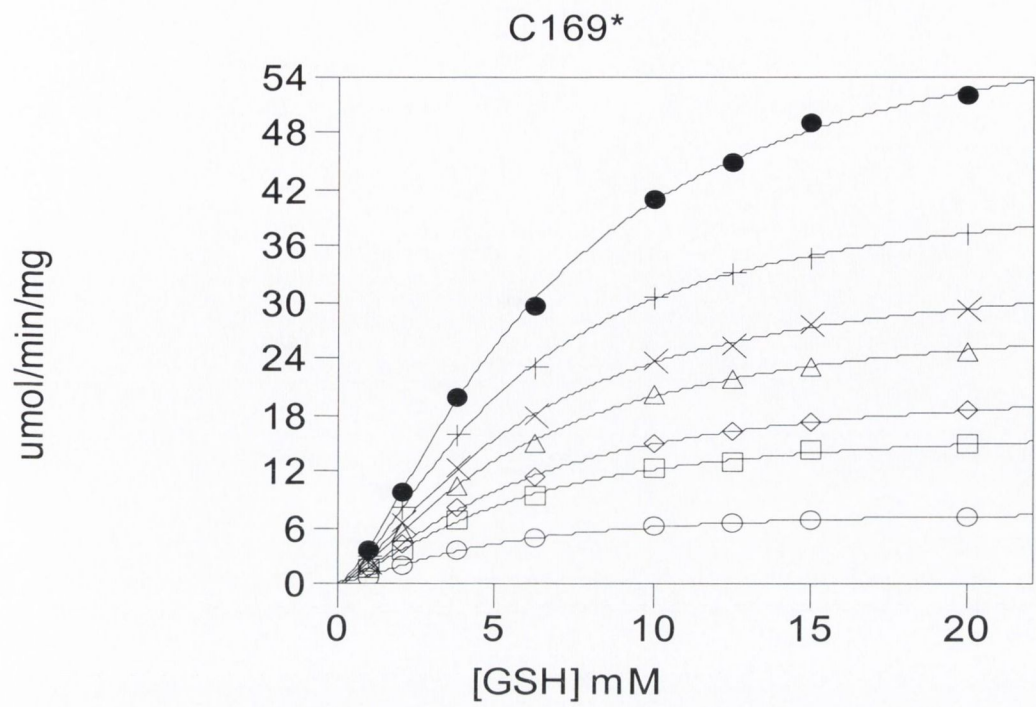
1.5mM CDNB

Km 5.52E-01  
Vmax 4.24E+01  
R<sup>2</sup> 9.97E-01  
n 1.22E+00

2mM CDNB

Km 5.15E-01  
Vmax 4.52E+01  
R<sup>2</sup> 0.996  
n 1.34E+00

Hill Plot



.2mM CDNB

Km 8.61E+00  
Vmax 8.25E+00  
R<sup>2</sup> 0.999  
n 1.37E+00

.4mM CDNB

Km 1.01E+01  
Vmax 1.73E+01  
R<sup>2</sup> 0.998  
n 1.38E+00

.6mM CDNB

Km 1.12E+01  
Vmax 2.17E+01  
R<sup>2</sup> 0.999  
n 1.39E+00

.8mM CDNB

Km 1.14E+01  
Vmax 2.95E+01  
R<sup>2</sup> 0.999  
n 1.38E+00

1mM CDNB

Km 1.20E+01  
Vmax 3.44E+01  
R<sup>2</sup> 9.99E-01  
n 1.41E+00

1.5mM CDNB

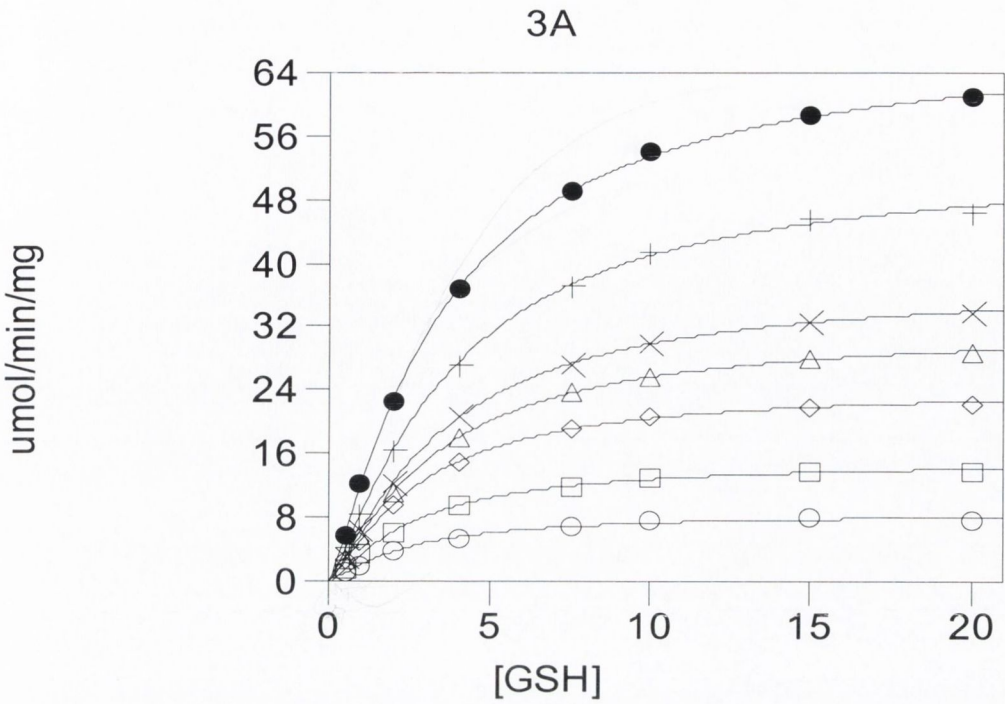
Km 1.24E+01  
Vmax 4.30E+01  
R<sup>2</sup> 9.99E-01  
n 1.47E+00

2mM CDNB

Km 1.49E+01  
Vmax 6.31E+01  
R<sup>2</sup> 0.999  
n 1.43E+00



Hill Plot



.2mM CDNB

Km 3.15E+00  
Vmax 8.60E+00  
R<sup>2</sup> 0.998  
n 1.27E+00

.4mM CDNB

Km 3.51E+00  
Vmax 1.52E+01  
R<sup>2</sup> 0.999  
n 1.28E+00

.6mM CDNB

Km 3.78E+00  
Vmax 2.43E+01  
R<sup>2</sup> 0.999  
n 1.31E+00

.8mM CDNB

Km 4.50E+00  
Vmax 3.19E+01  
R<sup>2</sup> 0.999  
n 1.27E+00

1mM CDNB

Km 4.85E+00  
Vmax 3.76E+01  
R<sup>2</sup> 9.99E-01  
n 1.27E+00

1.5mM CDNB

Km 5.24E+00  
Vmax 5.29E+01  
R<sup>2</sup> 9.99E-01  
n 1.26E+00

2mM CDNB

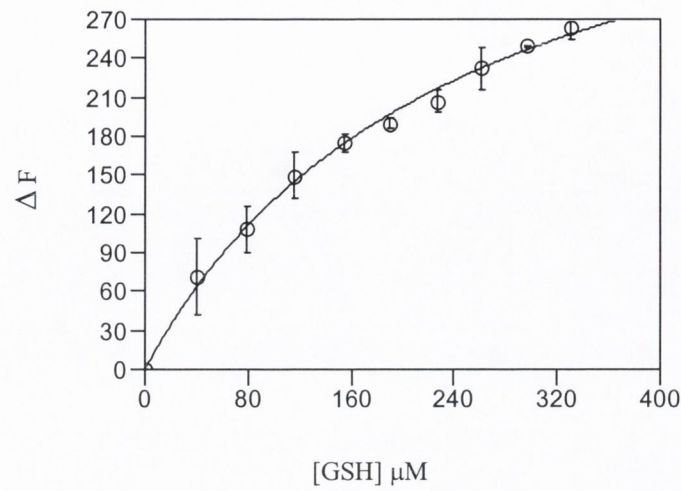
Km 4.64E+00  
Vmax 6.83E+01  
R<sup>2</sup> 0.999  
n 1.23E+00

## Appendix II

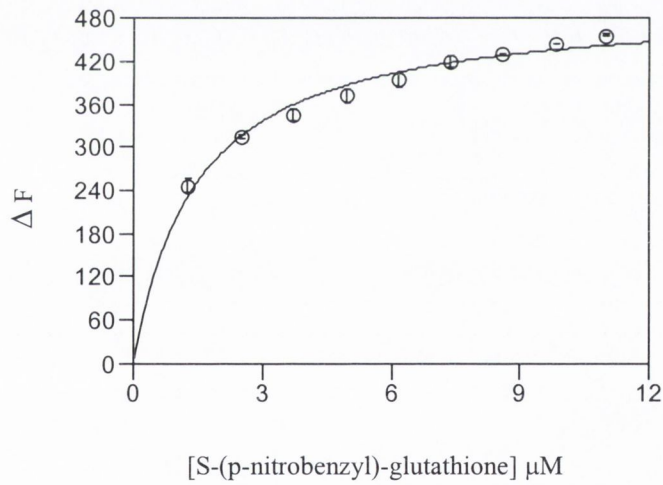
This appendix contains the fluorescence quenching for mGSTP1 and the C14A, C47A, C169A, C14\*, C47\*, C169\* and AAA mutants. The data is presented and fitted as hyperbolae for glutathione and S-(p-nitrobenzyl)-glutathione binding.

**mGSTP1-1**

(a)



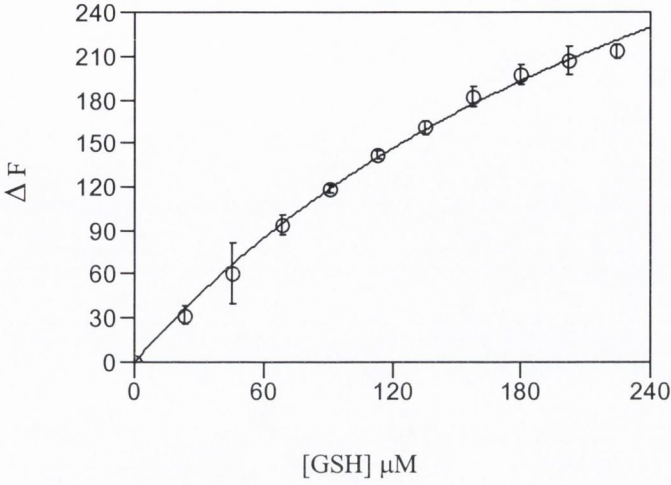
(b)



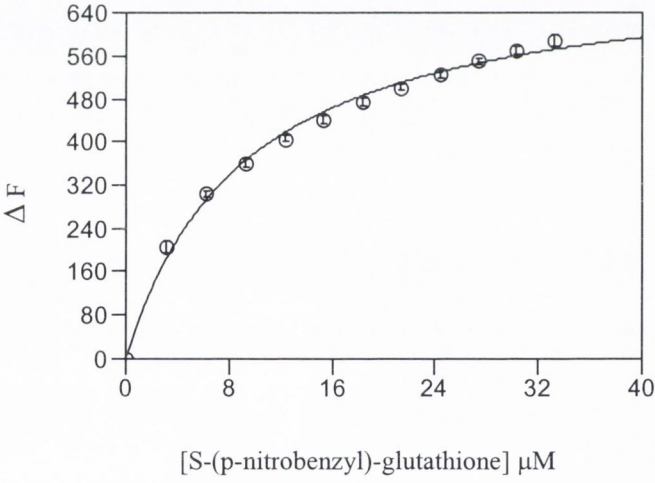


C14A

(a)

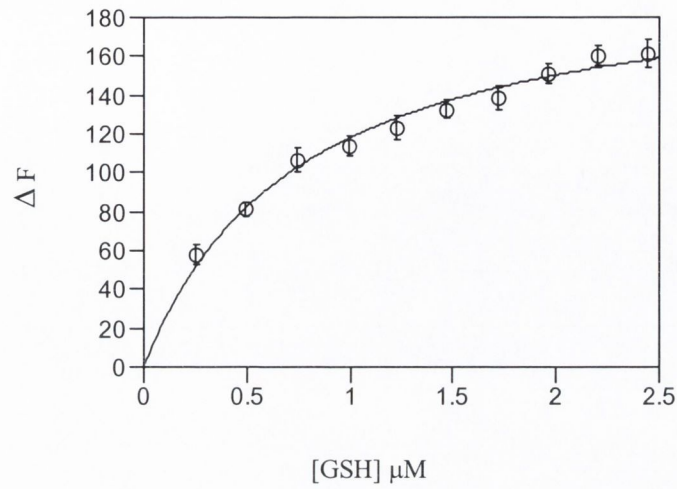


(b)

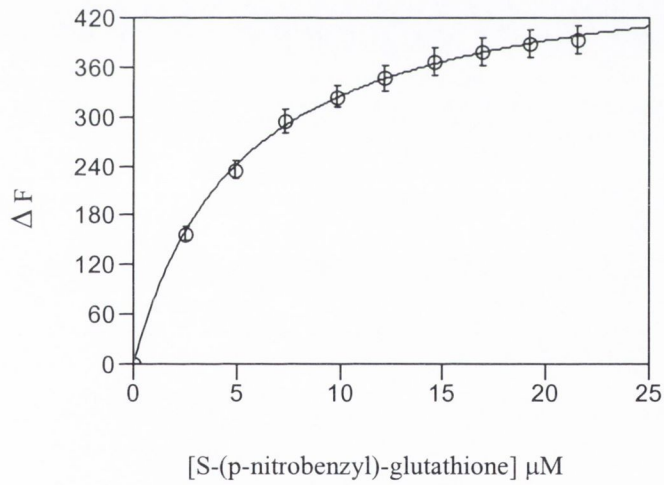


C47A

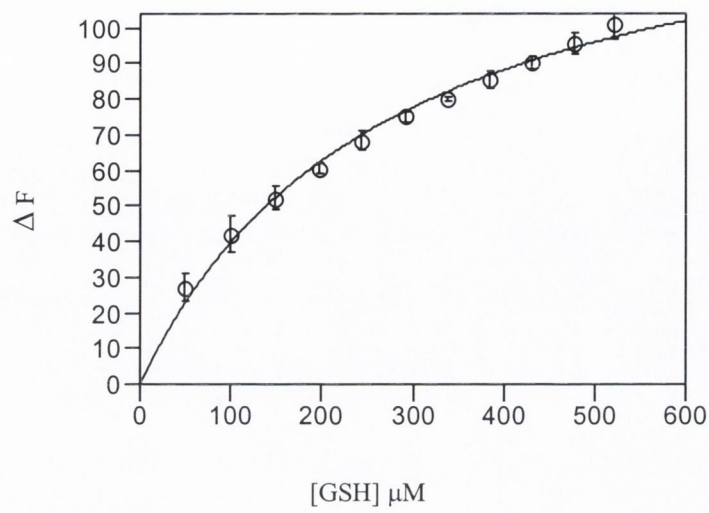
(a)



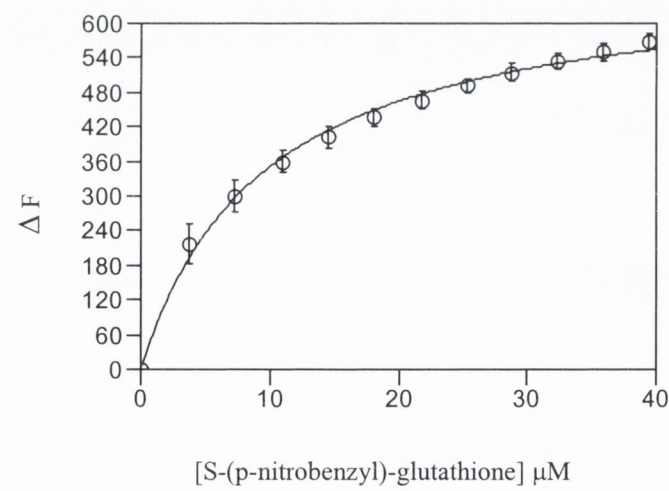
(b)



C169A



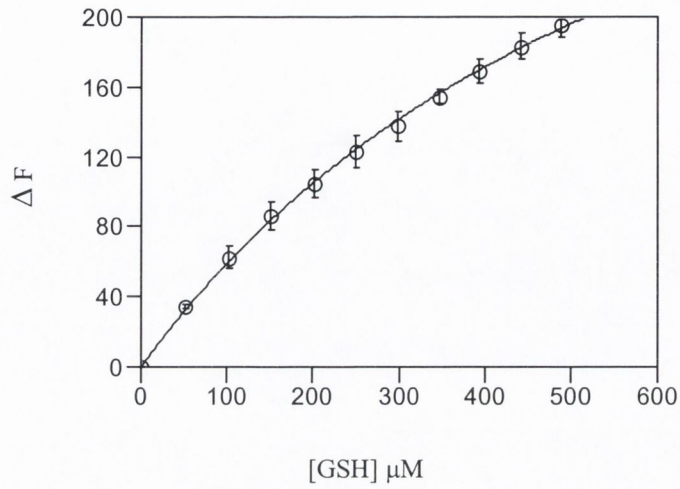
(b)



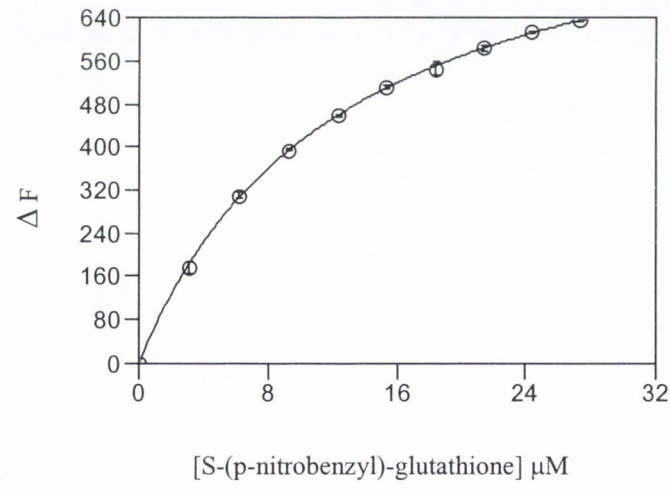


C14\*

(a)

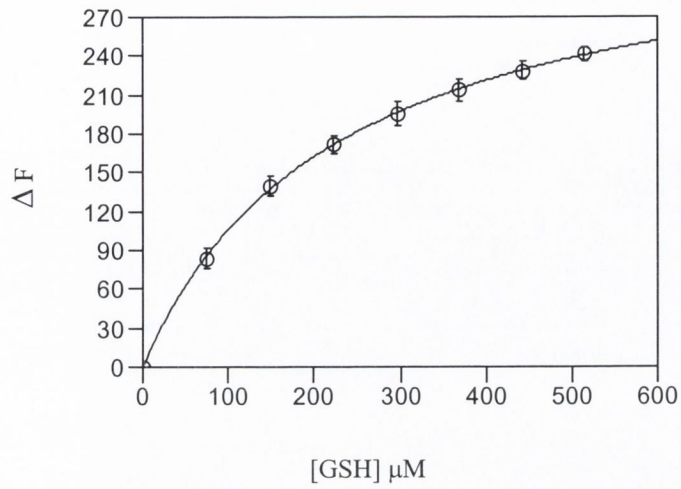


(b)

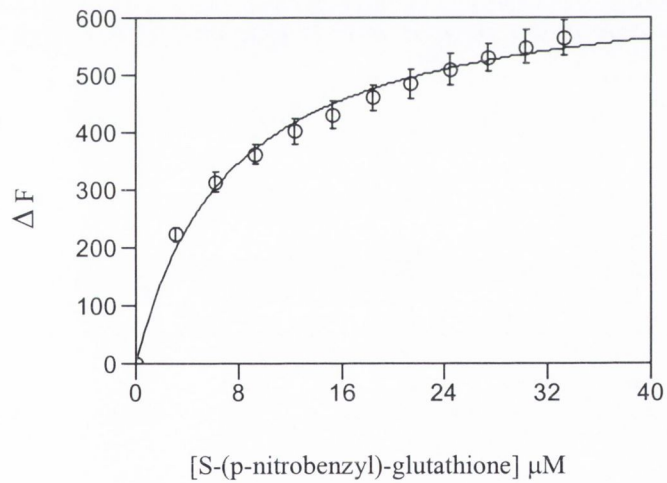


**C47\***

(a)

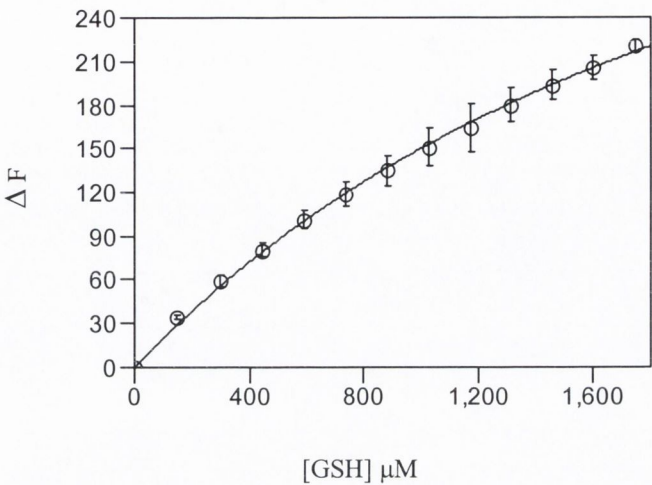


(b)

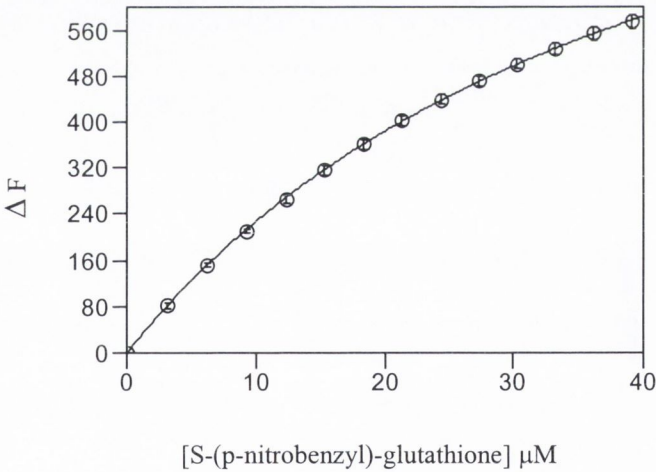


C169\*

(a)

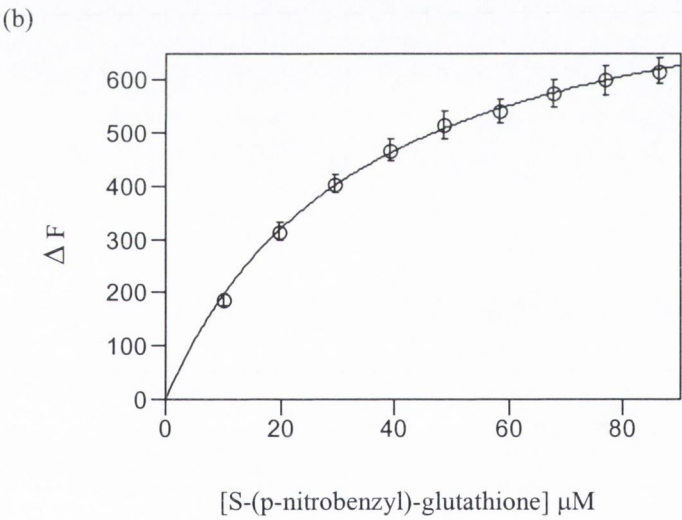
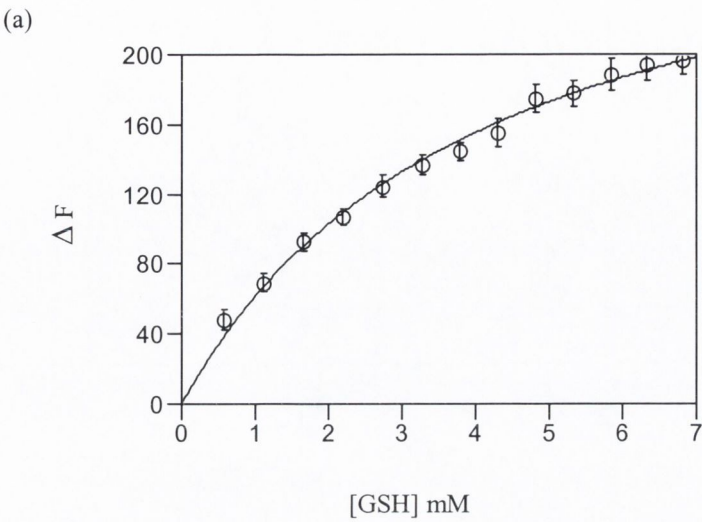


(b)





3A



**Phenotypes of loci and genes relevant to *E. coli* strains described in Table 2.1, Chapter 2.**

| Locus          | Gene             | Phenotype                                                                                               |
|----------------|------------------|---------------------------------------------------------------------------------------------------------|
| <i>ara</i>     |                  | arabinose utilisation                                                                                   |
|                | <i>araB</i>      | ribulokinase                                                                                            |
| <i>end</i>     | <i>endA</i> *    | DNA-specific nuclease I                                                                                 |
| <i>gal</i>     |                  | galactose utilisation                                                                                   |
| <i>gyr</i>     | <i>gyrA</i>      | DNA gyrase<br>subunit A, resistant to nalidixic acid                                                    |
| <i>hsd</i>     |                  | Host specific restriction and/or modification                                                           |
|                | <i>hsdM</i> **   | DNA methylase M                                                                                         |
|                | <i>hsdR</i> ***  | endonuclease R                                                                                          |
|                | <i>hsdS</i> **** | specificity determinant for <i>hsdM</i> and <i>hsdR</i>                                                 |
| <i>lac</i>     |                  | lactose biosynthesis                                                                                    |
|                | <i>lacI</i> ^    | repressor protein                                                                                       |
|                | <i>lac Z</i> ^^  | β-galactosidase                                                                                         |
| <i>lon</i> *** |                  | ATP-dependent protease L <sub>a</sub>                                                                   |
| <i>pro</i>     |                  | proline biosynthesis                                                                                    |
|                | <i>proA</i> **   | γ-glutamyl phosphate reductase                                                                          |
|                | <i>proB</i>      | γ-glutamyl kinase                                                                                       |
| <i>rec</i>     | <i>recA</i> ^^^  | general recombination and radiation repair<br>major recombination gene,<br>also involved in λ induction |
| <i>rel</i>     | <i>relA</i>      | regulation of RNA synthesis<br>ATP:GTP 3' pyrophosphotransferase                                        |

|            |                            |                                                                                                        |
|------------|----------------------------|--------------------------------------------------------------------------------------------------------|
| <i>rps</i> | <i>rpsL</i>                | small ribosomal protein<br>30S ribosomal subunit protein S12, may<br>confer resistance to streptomycin |
| <i>sbc</i> | <i>sbcB</i> *              | suppressor of <i>recBC</i><br>exonuclease I, involved in Rec F pathway                                 |
| <i>sup</i> | <i>supE</i><br><i>supF</i> | suppressor<br>amber (UAG) suppressor, inserts glutamine<br>amber (UAG) suppressor, inserts tyrosine    |
| <i>tet</i> |                            | tetracycline sensitivity or resistance                                                                 |
| <i>thi</i> |                            | thiamine biosynthesis                                                                                  |
| <i>tra</i> | <i>traD</i>                | suppresses conjugal transfer of F plasmids                                                             |
| <i>trp</i> |                            | tryptophan biosynthesis                                                                                |

\* The *endA1* mutation improves plasmid yield, especially from minipreps.

\*\*DNA methylase M is the modification subunit of the *EcoK* restriction-modification system. An *hsdM* strain is  $rk^+ mk^-$ .

\*\*\*Endonuclease R is the restriction subunit of the *EcoK* restriction-modification system. An *hsdR* strain is  $rk^- mk^+$ .

\*\*\*\*The HsdS gene product is the site recognition subunit of the *EcoK* restriction-modification system. An *hsdS* strain is  $rk^- mk^-$ .

\*Strains that are *recBC sbcB* display decreased rearrangement of cloned palindromic sequences, but are relatively slow growing.

\*\* The  $\Delta(lac-proAB)$  deletion removes the *lac* operon and surrounding region, including the two genes involved in proline biosynthesis. Most  $\Delta(lac-proAB)$  strains carry the deleted genes on an F' episome. When maintained on Minimal Medium the F' [*proAB*] genes provide positive selection for cells that retain their F' status.



^Most strains used with vectors that employ *lac* selection are *lacI<sup>q</sup>*. This mutation results in over-production of the *lac* repressor, minimising the low level of *lac* expression that occurs in uninduced wild-type cells.

^^The *lacZΔM15* deletion removes the amino-terminal α-peptide (amino acids 11-41) of β-galactosidase. Most cloning vectors that employ *lac* selection carry a gene that codes for the α-peptide and rescues the *lacZΔM15* mutation by α-complementation.

^^^A *recA* strain is recombination deficient and has a reduced ability to multimerise plasmids and rearrange cloned sequences. However, these strains grow relatively slowly, and have low transformation frequencies.

\*\*\*The *lon* mutation results in increased stability of fusion proteins, but results in a mucoid phenotype.